The role of APP and its homologous proteins in synaptic function and plasticity

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Abstract

For retrieval of memories a minimum number of synapses representing parts of the information is necessary. The dementia Morbus Alzheimer is characterized by progressive dysfunction and loss of synapses. Therefore, the central issue in understanding the genesis of the disease is the search for the underlying reasons of both. As the APP protein plays a central role in the pathogenesis of AD, the goal of this work was to elucidate the contributions of APP in synapse function in the hippocampus, the brain region that is predominantly affected by Alzheimer´s Disease.
Investigation of gene-targeted mice that expressed different truncations of APP, partly on an APLP2 knock-out background, allowed me to study the functions of the three major domains of APP in an in vivo system with physiological concentrations and processing of APP. All experiments were performed on the CA3-CA1 synapse in the mouse hippocampus by analysis of field recordings under various stimulus paradigms.

Mice lacking APP were found to display a deficit in long-term plasticity (LTP) that involved altered function of inhibitory synaptic transmission. This deficit was rescued in mice expressing the soluble ectodomain of APP (APPsα) instead of APP, showing that sustainment of intact function of LTP in old age is depending on and mediated by APPsα. The corresponding domain of the other relevant protein of the APP family, APLP2, revealed a minor role for synaptic plasticity.
With regard to the intracellular domains of both proteins, AICD and ALICD, a compensatory effect was observed. Simultaneous lack of functionality in both resulted in complex alterations of short-term plasticity, most prominently in enhanced responses to paired stimuli in the time-scale of 10 − 160 ms. They were likely mediated by involvement of two functions, intracellular Ca^{2+} buffering and inhibitory signaling. Assertions on the relative contributions and causation require further experiments. Whereas the effects of APPsα seemed to have a predominant relevance for unimpaired aging, the effects of the intracellular domains were independent from age.
The Aβ fragment, on the other hand, did not show a relevant role in synaptic plasticity. Neither long-term potentiation nor long-term depression were found to be altered by lack of this fragment in this experimental setup, focussing on physiological conditions. Compensation by simultaneous lack of possibly counteracting APPsα could be excluded. This revealed that unlike in a disease-related context, Aβ plays a minor role in the intact organism and also, that in initial stages of AD, the simultaneous lack of APPsα might be of higher relevance.

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1 Introduction

The memories we accumulate form the basis of our personality because they link our past to our future. They are individual informations about prior experiences, our thoughts and feelings that we have stored and that influence all future actions. But more than that, we need our memories to build concepts of our future. Based on our experiences we make predictions about future events and imagine different scenarios. Both are core attributes of a coherent personality and if memory declines, not only informations are lost, but to the same extent the identity vanishes.
It was recently shown that not only the storage and reactivation of memories but also the process of making predictions about future events is mediated by the hippocampi, structures of the mammalian forebrain (Squire, 2006; Schacter et al., 2007). That these processes are based on the same mechanisms is shown by the poverty of demented people not only in recalling past events, but also in imagining future scenarios (Schacter et al., 2007). Both, storage and recall of information involve unimpaired activation of neuronal circuits and restructuring of network connectivity, manifest as reweighting of synapses and reconstruction of neuronal connections. Therefore, every alteration that intervenes with the functioning of the involved mechanisms will have an impact on memory function both in a retrospective and in a prospective context.

The focus of my work was to elucidate the roles of the Amyloid Precursor Protein APP in synaptic function and synaptic plasticity. APP seems to hold a core role in the genesis of Alzheimer's Disease (AD), a disease that primarily affects memory function and is characterized by hippocampal synaptic dysfunction from the earliest stage on. Therefore, APP may also hold a vital role in synaptic functioning in the intact organism. So, to understand the genesis of Alzheimer's Disease, it is necessary to understand the involvement of APP in the functioning of synapses. At the same time, comprehension of the actions of APP on synaptic function in general may serve to elucidate its role in maintaining intact functioning of neuronal circuits throughout a lifetime. I set out to specify the function of APP in the intact organism by investigating the role of APP, its active fragments and homologous proteins in synaptic function and synaptic plasticity in the hippocampus under non-pathological conditions.

1.1 Synaptic plasticity

In order to incorporate and process new information, a neural network needs to be modifiable, or in the technical term: "plastic". A primary locus of plastic alterations is the synaptic connection between individual neurons. Modification of the strength of a synapse results in modification of information transmission. Important determinants are the amount of neurotransmitter released at the presynaptic (sending) side in response to an action potential and the amount and readiness of receptors proteins at the postsynaptic (receiving) side. To alter the amount of information flow, the synaptic strength needs to be increased or decreased. Donald Olding Hebb contributed to the search of the mechanism underlying activity-dependent plasticity of neuronal circuits by in his now famous postulate in which he demanded that the information in a network and thus also the locus of alterations should be in exactly those contacts between nerve cells:

> "When an axon of cell A … [exites] cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells so that A´s efficiency as one of the cells firing B is increased." (Hebb, 1949)

This statement comprises all necessary factors for an important form of activity-dependent synaptic plasticity, for long-term potentiation of synaptic strength. Most importantly, it states the fact that specific neuronal activity, or in other words, flow of information, is required to inflict alterations upon a neuronal network.
Therefore, these alterations are a necessary prerequisite for learning, as during learning, new information is taken up and has to be put in relation to already stored information or to new information, coming in at the same time. This links synaptic plasticity inseparably to learning processes. Naturally, information flow in an intact, behaving animal is very varied, comprising all kinds of successions of information coded as action potentials, which opens a wide field of parameters. This demands a reductionist approach to study the basic mechanisms underlying inter-cellular and intra-cellular events.

1.1.1 The hippocampus as a model

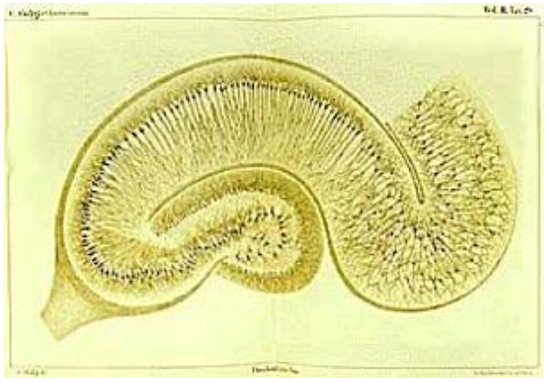
The hippocampi are evolutionarily old parts of the cerebral cortex, lying bilaterally in the depth of the cerebral hemispheres. In humans, the hippocampus assumes a central role in the processing of declarative memory, the form of knowledge that comprises facts about the self and the surrounding world in contrast to procedural memory comprising information about motion sequences. Famous cases of hippocampal lesions, as the case of the patient H.M., impressively show the role of the hippocampus in memory-related functions and for an individual´s personality. After a bilateral hippocampectomy, H.M. suffered from permanent

anterograde amnesia, not being able to incorporate new information into his knowledge (Scoville and Milner, 1957). This virtually arrested his personality at the time-point of the surgery. Very old informations, however, could be retrieved. His case thus demonstrates another key feature of the hippocampus. It serves for processing memory, interconnecting pieces of information and preparing them for later independent long-term storage in other, namely higher cortical brain regions.

The hippocampi receive inputs from the amygdalae, thalamus and higher cortical regions and process them by reconnecting them before neuronal activity is fed back in a modified way into these brain regions. Modifications take place during the predominantly unidirectional flow of information through the hippocampal formation.
The hippocampus has a lamellar structure, with axons running along a transverse plane. Within lamellae, pyramidal cells are aligned unidirectionally. The early schematic drawing in figure 1.1 A accentuates the regular alignment of pyramidal neurons. The somata of pyramidal neurons are located within an easily detectable band within area CA3 and CA1, the *stratum pyramidale*. They possess a shorter basal dendrite reaching into the *stratum oriens* on the "outer side" of the hippocampus and complex apical dendrites within the *stratum radiatum*, receiving feedforward information from pyramidal neurons of the ipsilateral hippocampus.

The hippocampal formation comprises the dentate gyrus (DG) and the ammon`s horn (cornu ammonis, CA). In the mouse, there are three subregions with distinct populations of excitatory neurons, forming a trisynaptic circuit between excitatory neurons (Fig. 1.1 B): the granular cells in the dentate gyrus, receiving incoming information, pyramidal cells of area CA3, receiving most input from the dentate gyrus via the mossy fibers plus direct information from other brain regions and the pyramidal cells in area CA1, receiving mainly input via the Schaffer collaterals from area CA3, but also from area CA1 of the contralateral hippocampus (Fig. 1.1 B).

A



B

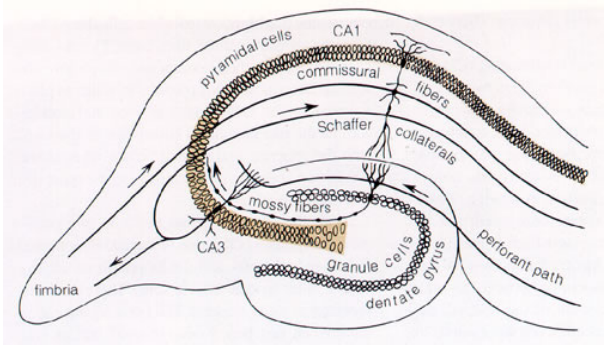


Figure 1.1 (A) Drawing by Camillo Golgi, 1903. **(B)** Schematic drawing of the hippocampal fiber connections, adapted from Hammond (2003).

Besides excitatory neurons, there are highly diverse populations of inhibitory interneurons in all subregions. Each area contains excitatory principal cells that project to the next area and a variety of interneurons mediating feed-back and feed-forward inhibition within an area and shaping rhythms of activity by grouped discharge that predisposes the network for alterations. In all subregions modification and interconnection of information takes place, before it leaves the hippocampus via the subiculum back to amygdalae, thalamus and cortex. At each of the stations connections can be altered in a Hebbian way.
Its highly organized network structure renders it an ideal model for investigation of specific synaptic contacts between groups of neurons. I investigated the hippocampal synaptic connection between area CA3 and CA1 as the standard model for investigation of long-term potentiation (LTP) and long-term depression (LTD). The present work was performed on the murine hippocampus. Also, in mice the hippocampi already assume a central role for learning and memory as behavioral studies after hippocampal lesions show.
D.O.Hebb already stressed that informations are not stored in single connections and single cells are not effective units of transmission (Hebb, 1949). The key concept of hippocampal functionality is that it temporally links neuronal activity representing aspects of information to assemblies. This assembly can still be active by reverberation after the offset of a stimulus combination by reverberating activity within that circuit. If these can be activated together again (and only then), the memory engram can be read out in its entirety. Hebb observed that this requires that the assembly can be made permanent.

1.1.2 Long-term potentiation

Such an assembly representing information is made permanent, if its connections are strengthened. This requires that the neuronal response to stimulation of a given intensity is enhanced. In 1973, such an enhancement was observed by Timothy Bliss and Terje Lømo. Applying a short train of high frequency pulses *in vivo* to a hippocampal pathway, they could observe a long-lasting enhancement of the response properties of the target neurons (Bliss and Lomo, 1973), a phenomenon that is now termed long-term potentiation (LTP).
Hebb´s postulate for the requirements for synaptic strengthening asks for coincident neuronal, i.e. pre-and postsynaptic activity and a subcellular mechanism of detection. In 1986, a realisation of this requirement was found. Wigström and Gustafsson showed the same potentiation of synaptic responses in a hippocampal slice preparation by simultaneously activating an axon bundle and a postsynaptic neuron (Wigström et al., 1986).

In many excitatory synapses a central role is assumed by the NMDA receptor, a ionotropic glutamate receptor, as shown by the prevention of LTP induction in area CA1 by application of a receptor antagonist (Collingridge et al., 1983). The NMDA receptor has a

permeability for Na^+ and Ca^{2+}, but is not involved in regular synaptic transmission, which is mediated solely by the ionotropic AMPA receptor, mediating Na^+ influx in response to glutamate binding. In response to regular synaptic stimulation the NMDA receptor does not open, due to occlusion of the channel pore by Mg^{2+} ions. Depolarisation of the postsynaptic terminal, however, releases the block and allows influx of Ca^{2+} (Herron et al., 1986). Elevation of the Ca^{2+}-concentration is the trigger for several intracellular reactions. This requirement of depolarisation demands simultaneous pre- and postsynaptic activity and renders the NMDA receptor the postulated "coincidence detector": only when the postsynaptic terminal is already or still active, i.e. depolarised and the Mg^{2+} block expelled while glutamate is released and binds to the NMDA receptor, it opens. This realises a key requirement of LTP: input specifity, stating that modifications of the synaptic network affect only the participating synapses. It was experimentally proven by intracellularly depolarising a neuron while applying single low frequency pulses to axons converging onto the neuron, which without postsynaptic depolarisation did not induce LTP (Wigström et al., 1986).

Bliss and Lømo could achieve the same effect by high-frequency stimulation, because the postsynaptic depolarisations caused by the individual stimuli summed up to reach the threshold for NMDA receptor activation. This mechanism ensures the second important characteristic of LTP: cooperativity. In order to confine plastic alterations to "outstanding" stimuli, representing either simultaneous information as parts of an active engram or strong inputs, these have to pass a certain threshold.

How are alterations confined to the active connection? On glutamatergic synapses, the postsynaptic elements are located on so-called dendritic spines, small membrane protrusions with bottle-neck connections to the dendrite. This, together with the passive signal propagation in dendrites, ensures that electric and ional alterations stay largely confined to the active part of the dendrite. The specificity, however, is not absolute. Part of the depolarisation spreads to nearby parts of the dendrite. This contributes to the phenomenon of cooperativity, permitting that inputs converging in close vicinity can be potentiated if they are active together, but it also explains the third feature of LTP: associativity. Also a weak input, that would alone not produce sufficient depolarisation, can be potentiated if it coincides with a stronger one, because the depolarisation from nearby active synapses helps to surpass the threshold of the NMDA receptor or of voltage-dependent Ca^{2+} channels. So, corresponding to Pavlovian conditioning, new engrams between previously unrelated stimuli can be formed, if one of them surpasses the induction threshold.

For comprehensive overviews on the mechanisms and significance of LTP see Bliss and Collingridge (1993), Malenka (2003), Citri and Malenka (2008).

The Ca^{2+} influx created by NMDA receptor activity triggers a cascade of events. Firstly, it triggers additional influx of Ca^{2+} into the cytosol from intracellular stores. The enhanced elevation of cytosolic Ca^{2+} levels can then activate different effector kinases, particularly

CaMKII, which phosphorylate AMPA receptors, thus prolonging their open probability, but also activate further signaling molecules leading to insertion of additional AMPA receptors into the postsynaptic membrane (Malinow and Malenka, 2002). This is demonstrated by the fact that blocking of kinase activity prevents LTP. Intracellular Ca^{2+} resting levels also influence NMDA receptor contributions. In a direct way, higher cytosolic levels lower the electrical and ional gradient which is the driving force for Ca^{2+}-influx. Indirectly, they lead to reduced opening probability of the receptor, because they trigger the activation of outward K^+ currents, leading to transient hyperpolarisation after stimulation. Both mechanisms are discussed as contributors to altered synaptic plasticity in Alzheimer's Disease and aging (Disterhoft et al., 1994).

Whether corresponding alterations on the presynaptic side are necessary, is still under debate and varies for different synapse types. At the CA3-CA1 synapse, most likely pre-and postsynaptic alterations take place. The induction of LTP occurs at the postsynaptic side, but then also retrograde signaling to the presynaptic side is effective. As candidate factors for diffusible retrograde messengers NO (Micheva et al., 2003) and BDNF (Gartner et al., 2006) have withstood critical experimentation. Besides that, in the hippocampus there are also "non-Hebbian" synapses, at which LTP is induced presynaptically and without NMDA receptor activation. The CA3-CA1 synapse, however, which was target of investigation in this thesis, is a typical "Hebbian synapse".
To incorporate alterations permanently into the network, additional functional and structural changes are necessary. These can comprise enlargement of spines or alterations of the postsynaptic scaffolding. Maintenance of LTP for longer than one hour requires protein synthesis or even alterations of gene transcription (Frey et al., 1988; Frey et al., 1989).

The components outlined here constitute the general mechanism at the hippocampal CA3-CA1 synapse, there are several other factors determining the actual shape of LTP. Depending on the protocol, involvement and kinetics of other ional channels differ, mostly depending on the temporal pattern on stimulation.

1.1.3 GABAergic contributions

Contributions of inhibitory neuronal activity are a critical determinant of hippocampal activity-dependent plasticity. In vivo, activity runs in waves through the hippocampal formation. These set the pace of the information flow and are shaped by phase-locked discharge of inhibitory interneurons releasing the neurotransmitter γ-aminobutyric acid (GABA). Thereby, interneurons strongly influence information transmission and also activity-dependent modulation of it.

During regular synaptic transmission a delicate balancing of excitation and inhibition is crucial, but it needs to be transiently tilted to allow for plastic changes. This is accomplished by the two different receptor systems for GABA. GABA acts on ionotropic $GABA_A$- and metabotropic $GABA_B$ receptors. $GABA_A$ receptors are predominantly located on synapses of inhibitory interneurons onto excitatory neurons, i.e. pyramidal cells in the hippocampal CA3 and CA1 region and mediate quick hyperpolarisation of the postsynaptic neuron by their association with Cl^- channels, whereas $GABA_B$ receptors are located pre- and postsynaptically, yet extrasynaptically and therefore only activate after spill-over of transmitter in response to strong activation. They activate with longer latencies and inhibit voltage-dependent Ca^{2+}- and K^+-channels (Hammond, 2003). Accordingly, $GABA_A$ receptors shape the temporal properties of regular synaptic transmission whereas $GABA_B$ receptors selectively act to enable synaptic plasticity during high-frequency stimulation. The NMDA receptor has a slow time constant of opening, resulting in slow rise time of NMDA receptor mediated EPSPs. Thereby, hyperpolarisation that is mediated by coincident inhibitory input prevents its opening. This ensures the specificity of the NMDA receptor for detection of simultaneous inputs.
Although blockade of GABAergic activity can facilitate NMDA receptor dependent LTP (Wigström and Gustafsson, 1983), in its natural form it is a key feature of LTP induction. After a single stimulus, the EPSP is curtailed by a feed-forward $GABA_A$ receptor mediated ISPC. During high-frequency stimulation, GABA accumulates and also stimulates extrasynaptic, metabotropic $GABA_B$ autoreceptors on GABAergic presynaptic terminals that inhibit subsequent GABA release (Davies et al., 1991). So, as stimulation continues, the release of GABA decreases, allowing enhanced opening of NMDA receptors. $GABA_B$ receptors have a peak of activity after 200 ms (Davies et al., 1990), finely tuning the system to suppress GABAergic inhibition during θ-type pattens of rhythmic activity, predominant during exploration and mnemonic processing (Macrides et al., 1982; Bland, 1986), which makes involved circuits especially receptible to modification.

1.1.4 Other forms of synaptic plasticity

The mechanisms of activity-dependent alterations of synaptic strength in the central nervous system and also the hippocampus are as different as the patterns of activity it receives. If an animal interacts with its environment, inputs of the most different temporal and local characteristics are converge on a neuron and cause interactive processes; likewise, requirements for storage vary greatly. LTP describes one model situation for activity-dependent synaptic plasticity.
Also, synaptic plasticity has to be bidirectional by nature, because if synapses were only potentiated, the information stored in them would ultimately be lost once all synapses had reached maximal strength. Therefore, equally important is the ability for lasting reduction of a

synaptic response, a phenomenon called long-term depression (LTD). An earlier notion was that LTD is a correlate of forgetting, but it is now undisputed that also modifications by LTD are directly involved in learning. The first observation that learning is associated with LTD was made by Manahan-Vaughan and Braunewell, reporting that under learning conditions LTD could be induced in rats (Manahan-Vaughan and Braunewell, 1999). A common theory, derived from observations in the developing visual system is that whereas LTP establishes the connectivities within a network, LTD accomplishes the fine-tuning of connections (Kemp and Manahan-Vaughan, 2007).

LTD was first described when Dudek and Bear found that prolonged low-frequency stimulation of the Schaffer Collaterals induces long-lasting reduction of synaptic responses (Dudek and Bear, 1992). The mechanisms mediating LTP were found to also be involved in the opposite phenomenon. Likewise, the consequences are opposing: AMPA receptors are dephosphorylized and internalized. Alterations are also initiated by activation of NMDA receptors, however by prolonged low-frequency (1Hz) stimulation. But as the temporal pattern of stimulation is different, so is the $[Ca^{2+}]_i$ elevation. Instead of quick steep increases of cytosolic Ca^{2+} levels, the increase is prolonged, but moderate. The crucial factor is that instead of activation of kinases this leads to activation of phosphatases, due to their higher affinity for Ca^{2+}. An important player is the phosphatase calcineurin or PP2B. Lisman proposed a model in which calcineurin mediates dephosphorylation of CaMKII, via activation of the protein phosphatase PP1 (Lisman, 1989). This theory was supported when it was reported that of calcineurin and PP1 can block LTD (Mulkey et al., 1993; Mulkey et al., 1994).
Shortly later, a form of LTD that was independent from NMDA receptor activation, but instead relied on activation of metabotropic glutamate receptors (mGluRs) was found (Bashir et al., 1993). This form of LTD is induced either by Schaffer Collateral stimulation with an only slightly different protocol, using 50Hz double pulses repeated at 1 Hz, or chemically by agonists of the mGluR (Schoepp et al., 1994).The mechanism of mGluR dependent LTD is much less clear. As it is blocked by Ca^{2+} chelators, its induction seems to rely on postsynaptic mechanisms (Bolshakov and Siegelbaum, 1994). However, it results in decreased frequency of miniEPSPs, not amplitudes, revealing a presynaptic locus of expression (Oliet et al., 1997).
Occlusion studies confirmed that both forms rely on independent induction mechanisms (Oliet et al., 1997) and are mutually exclusive (Bortolotto et al., 1999). NMDA receptor dependent LTD is readily induced in juvenile rodents, but not in adult animals. This allows the assumption that its main task is to refine the young neural network by targeted pruning of less significant contacts during late phases of development. Mechanistically, this is most likely due to changes in the subunit composition of NMDA receptors from NR2A- to NR2B-dominated forms (Okabe et al., 1998), altering the open time of the receptor (Erreger et al., 2005). mGluR dependent LTD is still inducible in the adult system (Kemp et al., 2000) and thus seems to be the mechanism in charge of keeping the balance of synaptic weights in the adult organism.

It is important to bring to mind that alterations like LTP and LTD are not digital phenomena, but are experimental paradigms representing the ends of a spectrum of alterations, localized on scales of stimulation parameters and physical reactions. Bienenstock, Cooper and Munro devised a model for net alteration of synaptic strengthes depending on the intensity of the stimulation (Bienenstock et al., 1982), which is adapted in figure 1.2 exemplarily for stimuli of different frequencies without simultaneous postsynaptic depolarization.

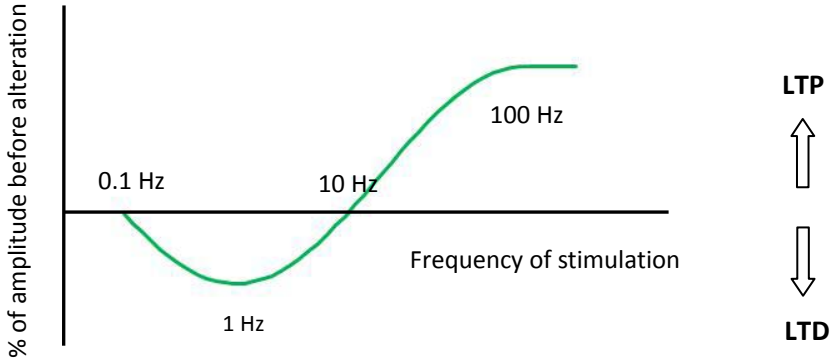


Figure 1.2 Induction of synaptic plasticity. The direction of change is dependent on the frequency of stimulation. Modified after Bienenstock, Cooper and Munro (1982). At 0.1 Hz, no plasticity-related processes occur. At 10 Hz, factors reducing and potentiating synaptic strength are balanced. At lower and higher frequencies LTD and LTP, respectively, are induced.

The level of potentiation in a group of neurons (or the probability of potentiation at a single neuron) is high for strong, i.e. high-frequency stimuli, reaches a balanced state at intermediate frequencies and results in the opposite alteration at low frequencies. 0.1 Hz represents the frequency that does not involve any plasticity-related processes. (The duration of stimulation which of course adds to the effect is disregarded here.) This shows that the outcome of the alteration is directly related to a continuum of stimulation intensities. A further consequence of this is that factors intervening with the strength of postsynaptic depolarization or other plasticity-related factors can shift the induction curve on the x-axis.

Also, in vivo, the wide spectrum of alterations results from various and possibly combined mechanisms that depend on the spatial and temporal pattern of activity a neuron experiences. Some combinations are exemplarily used to investigate certain aspects of alterations. The following forms of plasticity can occur at the CA3-CA1 synapse independently from or together with LTP. The term short-term potentiation is used for a form of synaptic plasticity that is NMDA receptor-dependent, but presynaptically expressed and dependent on the frequency of induction as well as subsequent activity (Volianskis and Jensen, 2003).
Paired-pulse facilitation (PPF) and post-tetanic potentiation on the other hand are NMDA receptor-independent forms of short-term plasticity that are typically presynaptically caused in the process of re-establishment of $[Ca^{2+}]_i$ levels after repetitive Ca^{2+} influx into the presynaptic

terminal. Both are also still inducible, when LTP is saturated. A consequence the multitude of influencing factors is that if a system is even only subtly altered by lack of a protein, this can entail several cascades of changed functions, shifting the balance of one or several contributing mechanisms to generate net effects that are in detail unforeseeable.

1.2 Alzheimer´s Disease

Alzheimer´s Disease (AD) is a disease that is clinically characterized by progressive impairment of cognitive functions, initially loss of the ability to retrieve memories, but later also further cognitive abilities like orientation, speech and abstraction. It was first described by Alois Alzheimer in his patient Auguste Deter (Alzheimer, 1907; Hardy, 2006). Nowadays, Alzheimer´s Disease is the most common of late-life dementias. Its prevalence increases with age, from roughly 2% of the 60 to 70 year old to up to 45% of the over 85 year old in the USA (Evans et al., 1989). This does not only make it the illness in the industrial world with the most quickly rising prevalence, but also it indicates that its etiology is strongly connected to age-dependent processes, even though it is not a part of normal aging.
In Alzheimer´s Disease (AD) usually the first recognizable and most prominent early symptom is the loss of declarative memory, depending on intact hippocampal synaptic function, before functions localized to other brain regions are also affected. Pathologically, in the final stage, AD is characterized by severe atrophy of the hippocampus and also cerebral cortex and, on the histological level, by amyloid plaques and neurofibrillary tangles. A protein that has gained much attention in connection with AD is Amyloid Precursor Protein (APP), because one of its naturally occurring cleavage products is β-amyloid (Aβ), which builds extracellular neurotoxic aggregates that assemble to amyloid plaques during the course of AD. Tangles are intracellular fibrils of hyperphosphorylated tau protein. The relevance of these aggregates for the disease is even less clear, probably reflecting axonal degeneration (Lee et al., 2001; Mandelkow et al., 2003).

For many years there was a controversy whether the amyloid plaques constitute the cause of memory and neuron loss or are rather consequence and a by-product of pathological alterations leading to these symptoms. It is noteworthy that the cognitive deficits found in AD (Scheff et al., 2005; Scheff et al., 2007) as well as in animal models (Rutten et al., 2005) occur much earlier than or even in the absence of neuronal degeneration. This has given rise to the conception that the reasons for AD lie in functional alterations at synapse and network level. And indeed, cognitive deficits strongly correlate with hippocampal and cortical synaptic dysfunction and amount of synapse degeneration (Terry et al., 1991; Masliah et al., 2006; Scheff et al., 2007), not with spatial and temporal patterns of plaque deposition and neuron loss

(Davies et al., 1987; DeKosky and Scheff, 1990; Terry et al., 1991). In animal models of AD, synapse loss and dysfunctionality also occur in regions without plaque formation or independently of plaques (Hsia et al., 1999; Mucke et al., 2000). Therefore now, increasingly, dysfunction and subsequent pruning of dendrites and loss of synapses are regarded as being the cause of the neuronal loss in later stages of the disease (Mattson and Chan, 2003) and the the focus of Alzheimer´s Disease research has shifted towards putative pathophysiological properties of soluble Aβ or small Aβ oligomers.
The initial events however, leading to AD pathology and putative involvement of other parts of APP are still largely unknown. Some studies on APP point to a role in synaptic function and neuroprotection, but the physiological role of APP and its other cleavage products in the intact nervous system is still mostly unknown. The hope is that an understanding of the mechanisms of how APP and its cleavage products act on synaptic function and plasticity under physiological conditions in the intact organism will lead to development of causal therapies as early as possible in the progression of the disease or even prevention.

1.3 Amyloid Precursor Protein

Amyloid Precursor Protein (APP) is a type I transmembrane protein which has been ascribed a multitude of putative and proven functions. The membrane bound holo-protein resembles a type I cell-surface receptor, comprising a large extracellular N-terminal part, a single transmembrane domain and a short intracellular C-terminal domain. The Aβ region lies partly in the transmembrane region (Glenner and Wong, 1984).
APP exists in different isoforms, APP_{770}, APP_{751} and APP_{695}, produced by alternative splicing (Kang et al., 1987; Golde et al., 1990). APP_{695} is the isoform that is predominant in the mammalian brain, expressed in neurons and differs from the two others, expressed in many other mammalian cells, by lack of a kunitz protease inhibitor (KPI) domain in the extracellular region (Turner et al., 2003; Zheng and Koo, 2006). In astrocytes the KPI domain containing isoforms seem to dominate (Gray and Patel, 1993; Rohan de Silva et al., 1997).
There are many indications that the amount of APP and its cleavage products may play a central role in the development of the disease. Hereditary forms of early onset Alzheimer´s Disease are concomitant with mutations in the *APP* gene or the *PSEN* gene, coding for a protein involved in the processing of APP. Also, the *APP* gene is located on chromosome 21 in humans (Kang et al., 1987) and persons with trisomy 21 almost invariably develop AD pathology (Wisniewski et al., 1985; Lai and Williams, 1989; Maatta et al., 2006).

Understanding of the role of APP in the mammalian organism is complicated by several facts. Firstly, APP can be subjected to different and mutually exclusive cleavage pathways

yielding products with specified functions in the organism and not all studies could clearly refer to cleavage products or the holo-protein. Also central to the processing of APP and ultimately for the understanding of AD is its localisation, as the different cleavage steps are performed in different compartments. Analysis of APP functions is further complicated by the fact that APP is part of an evolutionarily conserved family of proteins, in mammals comprising two APP-like proteins, the APLPs, resuming partly overlapping functions.

1.3.1 APLP Protein family

APP is part of an evolutionarily highly conserved gene family, comprising APPL in *Drosophila*, APL-1 in *C.elegans* and in mammals the amyloid-precursor-like proteins APLP1 and APLP2 (Slunt et al., 1994; Coulson et al., 2000). These are largely homologous to APP, see figure 1.3, the only difference being that they do not contain the sequence encoding for the Aβ domain and therefore cleavage of APLP1 and APLP2 does not set free Aβ (Zheng and Koo, 2006), but an AP-1 and AP-2 fragment without known functions (Walsh et al., 2007). Nevertheless, they are spliced and and processed in an identical manner (Scheinfeld et al., 2002; Eggert et al., 2004; Li and Sudhof, 2004; Endres et al., 2005).

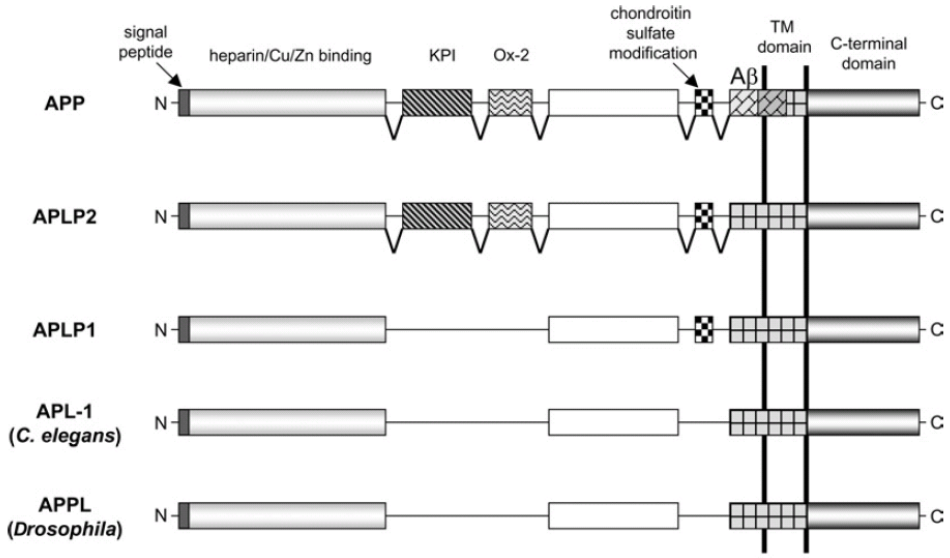


Figure 1.3 The family of APP proteins contains in mammals, APP, APLP1 and APLP2, in C.elegans APL-1 and in Drosophila APPL. All members contain a signal peptide sequence, a tansmembrane domain and a C-terminal domain containing a NPXY motif. In non-neural cells, APP and APLP2 isoforms containing several domains in the extracellular part are generated by alternative splicing. In neurons, forms without the indicated domains are expressed. Only the transmembrane (TM) domain of APP contains the Aβ region. Adapted from Walsh et al. (2007).

The homology is paralleled by overlapping and partly redundant functions. APP and APLP2 are ubiquitously expressed in the organism (Slunt et al., 1994) and ablation of both APP and APLP2 in mice results in early postnatal death (Heber et al., 2000). APLP1, on the other hand, is restricted to the nervous system and APP/APLP1 combined knock-out mice are viable (Anliker and Mueller, 2006). Differences also occur in the subcellular expression pattern: APP and APLP2 are found to be enriched at presynaptic sites (Lyckman et al., 1998; Lazarov et al., 2002), whereas APLP1 is present at postsynapses (Kim et al., 1995).

1.3.2 Cleavage pathways and products

Amyloid Precursor Protein (APP) has long been in the focus of research because its cleavage can yield Aβ. The key to an understanding of the multiple effects of APP is the processing of APP, because generation of the functional fragments is partially mutually exclusive. The cleavages themselves occur constitutively, but can be regulated. A multitude of factors alters the cleavage balance and consequently the balance of cleavage products and their effects on the organism. Thus, every alteration of the balance between these two pathways will entail a multitude of effects. Such alterations can be effected by regulation of the activity of the cleavage enzymes (secretases), but also by alterations of the intracellular transport of APP, as the different proteases are located in different intracellular compartments.
As more became known about the functions of the different fragments of APP, the question arose whether alterations in the respective amounts may contribute to the cognitive dysfunctions occurring in Alzheimer´s Disease (Mattson, 1997; Turner et al., 2003). It is noteworthy here, that APP processing changes profoundly with aging and the development of AD. So-called non-amyloidogenic cleavage is reduced in favour of cleavages that set free Aβ and preclude the generation of another important product, APPsα (Lannfelt et al., 1995; Nistor et al., 2007). The levels of the cleavage products mirror the state of cognitive functions (Almkvist et al., 1997), demonstrating the relevance of this change.

The first step is cleavage either by α-secretase or by β-secretase (Fig. 1.5, next page). This step decides about later generation of Aβ, because whereas the β-secretase cleaves at the N-terminal end of the Aβ region (Li and Sudhof, 2004; Vassar, 2004), α-secretase activity precludes generation of intact Aβ, cutting within the Aβ region (Allinson et al., 2003). These steps release the largest part of the APP ectodomain (APPsα or APPsβ respectively) and leave a short membrane-anchored C-terminal fragment of either 83 or 99 animo acids. In a second, intramembranous step, both fragments are processed by γ-secretase, cleaving at the C-terminal end of Aβ. After earlier β-secretase cleavage this releases the Aβ fragment and the APP intracellular domain (AICD). In the case of antedecent α-secretase cleavage, the AICD and the so-called p3 fragment are generated. Therefore, inhibition of γ-secretase cleavage (Dominguez

et al., 2004) as well as enhancement of α-secretase cleavage (Etcheberrigaray et al., 2004; Fahrenholz and Postina, 2006) are discussed as new therapeutic targets.

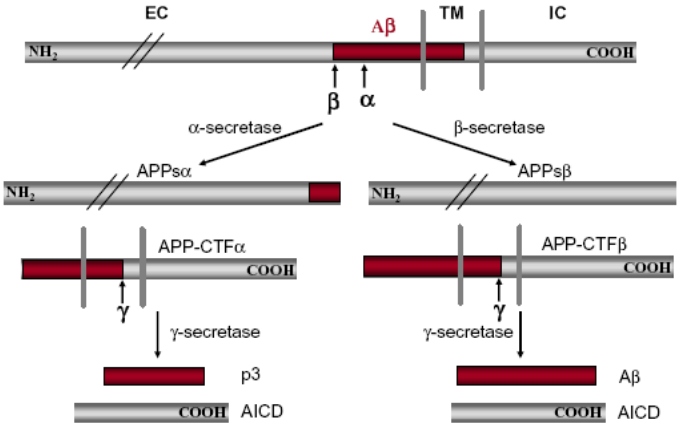


Figure 1.5 APP is subjected to two partially exclusive cleavage pathways. In a first cleavage step the majority of the N-terminal ectodomain is shedded. Shedding by α-secretase releases the APPsα fragment and precludes generation of intact Aβ. Cleavage by β- and subsequent γ-secretase generate Aβ and the AICD, the latter also being generated if γ-secretase cleavage follows α-secretase activity. Adapted from Zheng and Koo, (2006).

γ-secretase can cleave at positions corresponding to amino acids 40 or 42 of Aβ. The two differ in their readiness to build oligomers and fibrils and ultimately give rise to amyloid plaques. Under normal conditions, only less than 10% of the Aβ generated are Aβ1-42, which aggregates more readily. In cases of mutations leading to early-onset AD, this relation is shifted in favour of Aβ1-42 (Gandy et al., 2001).

Table 1.1 Overview of the nature and substrates of neural APP secretases in mammals.

Neural secretases of APP	Proteins	Cleavage products of APP	Further substrates
α-secretases	ADAM9	APPsα	APLP1
	ADAM10	CTFα	APLP2
	ADAM17		N-Cadherin
	BACE2		$P75^{NTR}$
	MDC9		
β-secretase	BACE1	APPsβ	APLP1
		CTFβ	APLP2
γ-secretase-complex	Ps1 + 2	Aβ	APLP1
	+ Nicastrin	P3	APLP2
	+ Aph-1	AP	Notch receptor
	+ Pen-2	AICD	

The identities of the three secretases are mostly clarified. Among the α-secretases are the zinc metalloproteinases ADAM9, ADAM10 and ADAM17 (TACE), MDC-9 and the

aspartyl-protease BACE2, reviewed in Thinakaran and Koo (2008). ADAM9, 10 and 17 additionally cleave N-cadherins (Maretzky et al., 2005), the EGF receptor and TNF-α (Deuss et al., 2008). ADAM 17 also cleaves the pan-neurotrophin receptor $p75^{NTR}$ (Weskamp et al., 2004). The transmembrane aspartyl-protease BACE1 is the major neural β-secretase (Vassar, 2004) and seems to be substrate-specific for APP. The γ-secretase is a large protein complex, its catalytic domain being formed by the presenilin PS1 and PS2 (Verdile et al., 2006). Mutations in PS1 and PS2 cause the most aggressive forms of early-onset AD (Sherrington et al., 1995; Levy-Lahad et al., 1995). For an overview of the APP secretases see Table 1.1.

So, a crucial determinant of APP function in a tissue is the size of the fractions of APP processed by either of the competing proteases. As the proteases are localised in different subcellular loci (reviewed in Small and Gandy (2006)), this is determined primarily by the amount of APP in different compartments. In non-neural cells, α-secretase activity was found to occur predominantly at or close to the plasma membrane (Sisodia, 1992; Parvathy et al., 1999), whereas Aβ generation takes place mainly in the trans-golgi and endosomes during secretory and recycling pathway after internalisation from the plasma membrane. β- and γ-secretase are localized in the golgi, trans-golgi and endosomes, γ-secretase additionally in the ER (Koo and Squazzo, 1994). Therefore, rates of intracellular transport, internalisation and sorting of APP are crucial for the balance of cleavage products and explain why inhibitors of APP internalisation can increase α-secretase cleavage (Pietrzik et al., 2002; Pietrzik et al., 2004; Russo et al., 2005). Also, only 10% of the cell´s APP is localized in the plasma membrane. The largest part of APP and also the α-secretases are on the way to the cell surface (Thinakaran and Koo, 2008), a process that can be modified by generation and release of more secretory vesicles.
Changes of internalisation are mediated by interaction with cytoplasmatic adaptor proteins and intracellular signalling. Clathrin-mediated endocytosis is mediated via interaction of the YENPTY motif (amino acids 682–687) on the APP intracellular domain with cytoplasmic adaptor proteins Fe65, X11 and Mint (Russo et al., 2005) and is phosphorylation-dependent (Pastorino et al., 2006; Sodhi et al., 2008). Endocytosis occurs rapidly and constitutively, but can be modulated by pathways involving protein kinase C, tyrosine kinases, mitogen-activated protein kinases (MAPKs) and extracellular signal-regulated kinase (Lee et al., 1995; Ulus and Wurtman, 1997) and via cAMP in astrocytes (Young et al., 1999). Activators of these pathways are muscarinic and epidermal growth factor receptors, that when activated, all enhance the production of APPsα (Ulus and Wurtman, 1997). Also, neuronal activity is regarded as influential modulator of the cleavage balance. However, observations on the consequences of increased neuronal activity are controversial.
On the one hand, electrical stimulation of the complete hippocampal slice was reported to cause rapid increase in the transmitter release and APPsα generation could be inhibited by the sodium channel blocker TTX, suggesting that action potentials mediate the release of APPsα and leading the authors to conclude that neuronal activity regulates APP processing by upregulating α-secretase acivity (Nitsch et al., 1993). APPsα release has been shown to occur

following the *in vivo* induction of LTP in the dentate gyrus of anaesthetized rats (Fazeli et al., 1994). Also, activation of metabotropic glutamate receptors has been shown to increase APPsα secretion via PKC-dependent mechanisms (Ulus and Wurtman, 1997) and NMDAR activation was reported to increase APPsα secretion in differentiating neural precursor cells (Gakhar-Koppole et al., 2008). Activity-dependent upregulation of ADAM17/TACE (Buxbaum et al., 1993) and transport of ADAM10 to the plasma membrane (Lammich et al., 1999) are also described. On the other hand, Kamenetz and colleagues observed that neuronal activity increased Aβ production in hippocampal slices overexpressing APP (Kamenetz et al., 2003). They postulated a neuroprotective negative feed-back loop by subsequent depression of NMDA repeptor dependent activity by Aβ. On the other hand, β-secretase activity has not been found be regulated (Lichtenthaler, 2006).

It has to be kept in mind, however, that most of the aforementioned findings refer to cultured non-neural cells. In neurons, much less is known about the locus of the processing steps. Due to their special morphology, comprising a complex dendritic arbour and a long axonal process both bioenergetically dependent from the perikaryon, processing is modified. In neurons, APP is transported axonally in a kinesin-I dependent way (Kaether et al., 2000) and undergoes retrograde and transcytotic transport to dendrites of neurons (Kim et al., 1995; Lyckman et al., 1998; Lazarov et al., 2002). It has been proposed that cleavage in neurons takes part during anterograde, axonal transport to the membrane (Buxbaum et al., 1998), but see also Parvathy et al. (1999). The major secretase in neurons seems to be β-secretase and neurons are believed to be the major source of Aβ (Thinakaran and Koo, 2007). There is evidence that at least part of Aβ is synaptically released (Koo et al., 1990; Lazarov et al., 2002).

1.3.3 Functions of APP

The question that emerges regarding the exclusivity of the cleavages and the diverse effects of the products is, whether the resulting APP fragments may have different, maybe even opposing effects in the organism. Such a construction would have far-reaching consequences for even subtle alterations of the cleavage balance and becomes interesting in the light of disbalanced amounts of cleavage products in AD, because it raises the possibility that the deficits in AD, at least to some extent, might result from impaired APP function in general, not only from elevated Aβ levels.

Most of the functions ascribed to APP circle round trophic and cell-death mediating actions. There is extensive literature concerning the intra- and extracellular functional domains of APP, however only seldom distinguishing between functions of APP as a holo-protein and of the cleavage products. Effects of APP on neurite outgrowth and synaptogenesis (Milward et

al., 1992; Gakhar-Koppole et al., 2008) as well as differentiation of neural stem cells (Sugaya et al., 2006; Kwak et al., 2006) have been described and led to the idea that APP acts via autocrine and paracrine signaling, reviewed by Thinakaran and Koo (2008) by shedding of the APP ectodomain. Also, anti-apoptotic and neuroprotective properties have been found, reviewed by Mattson and Chan (2003). Decreased levels of APPsα are associated with deficits in CNS function in AD and aging (Lannfelt et al., 1995; Nistor et al., 2007), which is consistent with observation that during aging and the development of AD profound changes in APP processing occur (Kern et al., 2006).
Another proposed role of APP is in cell adhesion, because APP colocalizes with integrins at adhesion sites (Storey et al., 1996) and has been shown to build homo- and heterodimers *in cis* and *in trans* with other members of the APP family (Soba et al., 2005). An interesting hypothesis concerning the role of APP as well as APPsα was put forward recently by Young-Pearse and co-workers. They supported the role of APP in controlling neurite outgrowth by binding to integrins, but also proposed a role of APPsα in preventing this by competitive binding to integrins (Young-Pearse et al., 2008).

Of all APP fragments, Aβ has received most of the attention, because of its aggregate-building properties in high concentrations. Aβ aggregates have been reported to have various effects on synaptic strength and structure. In models of overexpression or exogenous addition of Aβ, it was shown to depress synaptic currents via action on AMPA and NMDA receptors (Kamenetz et al., 2003; Snyder et al., 2005) and alter pre- and postsynaptic structure of neurons (Roselli et al., 2005; Calabrese et al., 2007). A surplus of Aβ oligomers could also negatively affect synaptic plasticity (Walsh et al., 2002a; Hsieh et al., 2006; Shankar et al., 2008). However, studies documenting a physiological role of Aβ are still lacking.

The function of the AICD also is under debate. Of particular interest here is again the YENPTY motif which is completely conserved from *C. elegans* to humans and binds to numerous proteins including X11/Mint, Fe65 and JIP, reviewed in Annaert and De Strooper (2002) and King and Scott Turner (2004). Because APP is cleaved by the same secretase as the Notch receptor after ligand binding, it has been proposed that after shedding, the AICD might build a transcriptionally active complex with Fe65 and Tip60 regulating gene expression (Cao and Sudhof, 2001; Cao and Sudhof, 2004). It was reported to induce expression of the Aβ degrading neprilysin, providing a negative feedback control on Aβ generation (Pardossi-Piquard et al, 2005). But also a role in gene transcription without prior γ-secretase cleavage has been shown (Hass and Yankner, 2005). An alternative view is that the AICD serves a more general signaling function and is involved in Ca^{2+} homeostasis, e.g. by triggering Ca^{2+} release form internal stores (Leissring et al., 2002) and maintaining stable Ca^{2+} levels in the cytosol by supporting transport of cytosolic Ca^{2+} into the endoplasmic reticulum (Hamid et al., 2007). Findings from PS1 overexpressing or KO animals indicate that this role might be attributed to APP processing and the cleaved AICD (Schneider et al., 2001; Herms et al., 2003).

1.4 Scope and intention

Alzheimer`s Disease is characterized by progressive dysfunction and loss of synapses in hippocampus and cerebral cortex and the notion is now prevailing that AD may initially be a diease affecting synaptic connectivity in these brain regions. The Amyloid Precursor Protein (APP) is not only critically involved in the pathogenesis of Alzheimer's Disease, it may also play an important physiological role for the maintenance of general functions in neurons and specific functions in synapses, especially during aging.

To understand the pathogenesis of Alzheimer's Disease, as well as to specify the role of APP in memory-related functions, investigation of its involvement in memory-related synaptic processes in the intact organism is necessary. Hereto, it is essential to apply a model system that does not imitate pathogenic mechanisms of AD by investigating disease-relevant mutations of APP or overexpressing parts of it. Also, in such systems the levels of the respective fragments are interdependent, which inevitably confounds any conclusion as to which fragment a given function can be attributed. Therefore, I performed my experiments on gene-targeted mice expressing physiological levels of truncated versions of normal APP in combination with deletion mutants for APP or its homologue APLP2. This allowed me to attribute certain alterations to distinct regions of APP without confounding them with the effects of others. Working in an *ex vivo* model also enabled me to study the consequences in a system of functional neural connectivity.

I focussed my examination on three major active fragments of APP: the soluble ectodomain APPsα, as liberated by α-secretase cleavage, the transmembrane region Aβ and the cytosolic domain AICD, both generated by β- and γ-secretase. To exclude potential masking effects of the APP homologous protein APLP2, I extended my studies to combined deletion mutants. If specification of age-dependent alterations was of relevance, I performed experiments on aged individuals. As object of investigation I have chosen the CA1-CA3 synapse in the mammalian hippocampus which serves as reference for most investigations of memory related synaptic functions. In this model, by means of analyzing field potentials under various conditions, I specified characteristics that are of relevance for intact synaptic transmission and probed for alterations in synaptic plasticity.

2 Materials and Methods

2.1 Transgenic Mice

2.1.1 Description of used mouse lines

I examined six strains of gene-targeted mice carrying deletions of the *APP* and *APLP2* gene or transgenes for fragments of the APP protein, all generated in the laboratory of Prof. U. Müller, Heidelberg. Table 2.1 gives an overview over the transgenes and the nature of the modifications.

Table 2.1 Strains of mice used in this work. (AS: amino acids)

Name	Description
$APP^{-/-}$	deletion of the *APP* gene
$APLP2^{-/-}$	deletion of the *APLP2* gene
APPΔCT15	replacement of APP by a truncated version lacking the last 15 AS
APPsα	replacement of APP by the soluble product of α-secretase cleavage
$APLP2^{-/-}$/APPΔCT15	deletion of the *APLP2* gene plus replacement of APP by a truncated version lacking the last 15 AS
$APLP2^{-/-}$/APPsα	deletion of the *APLP2* gene plus replacement of APP by the soluble product of α-secretase cleavage

$APP^{-/-}$ animals had been generated with a strategy based on the P1-sequence-specific recombination system. For deletion of a gene, *loxP* sequences are inserted upstream and downstream of the target gene by homologous recombination. After transfection with a cre recombinase expression plasmid, the P1 cre recombinase performs a recombination between *loxP* sites and deletes the sequence flanked by the *loxP* sites. Here, in embryonic stem cells *loxP* sequences had been inserted into the *APP* gene in a way that introduction of a *loxP* targeting vector and cre expression plasmid by electroporation deleted codons 72 to 737. The positive clones had then been microinjected into the blastocysts of C57/Bl6, which gave rise to germline-transmitting chimeras. Intercrosses of heterozygous $APP^{-/-}$ mice led to homogzygous

mutant animals (Li et al., 1996). APLP2$^{-/-}$ mutants had been generated by deletion of the promoter and first exon of *APLP2* with an inactivation vector (von Koch et al., 1997).

For the APP transgenic mouse strains carrying deletion mutants of *APP*, knock-in (KI) lines were generated that under control of the endogenous *APP* promoter express either exclusively a sequence coding for a truncation identical to the secretable fragment, generated by α-secretase cleavage ("APPsα") or a C-terminal deletion mutant lacking the last 15 amino acids of APP harboring the intracellular YENPTY interaction motif ("APPΔCT15") (Fig. 2.1 A).

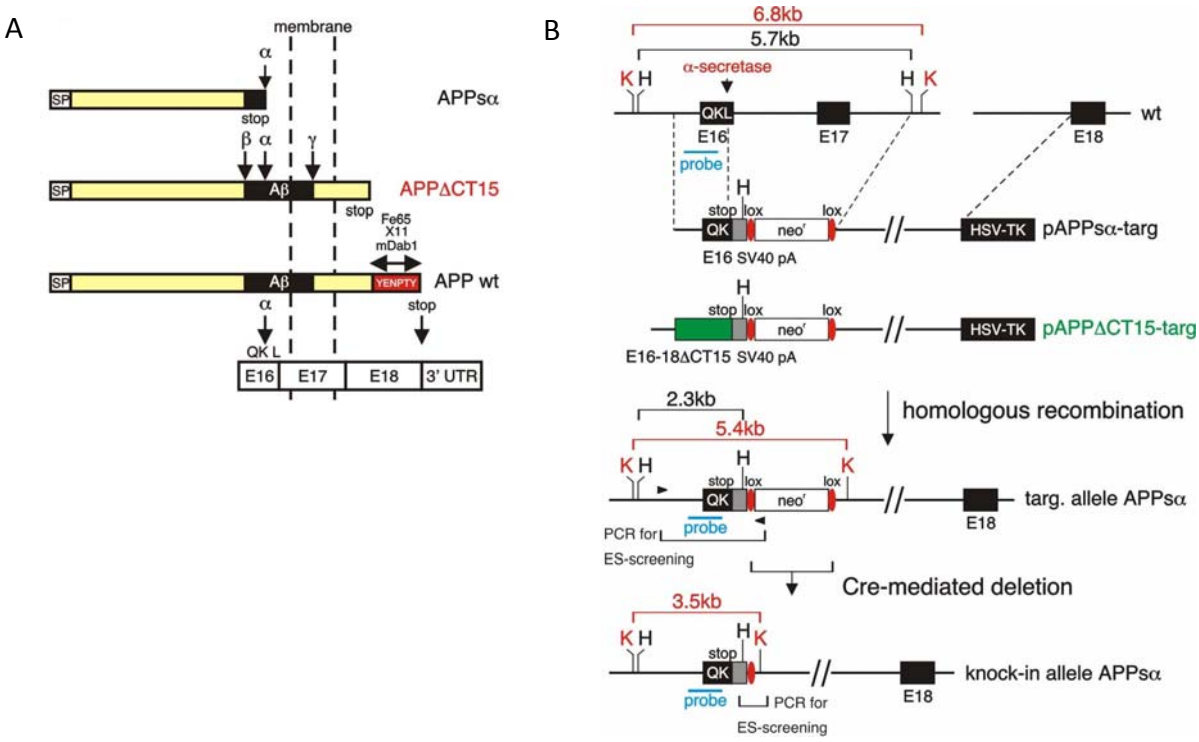


Figure 2.1 Generation of knock-in mice. **(A)** Schematic representation of the sequences of the APP variants APPsα and APPΔCT15. **(B)** Schematic representation of the knock-in strategy. For generation of APPsα expressing mice, a stop codon was introduced behind the α-secretase site at exon 16. In the pAPPΔCT15-targ vector the coding sequence of exon 16 was fused to APP$_{695}$-cDNA sequences up to amino acid 680 flanked by a stop codon. The stop codon was followed by a SV40 polyA site and a floxed neo^{R} gene for selection in ES cells. Subsequently, the neo^{R} gene was deleted by transient expression of cre-recombinase (**B**, bottom). Adapted from Ring et al. (2007).

For both lines, a stop-codon had been introduced into *exon 16* behind the α-secretase site at position QK612 on the *APP* locus using a replacement type targeting vector. For generation of APPΔCT15-KI mice, additionally, the coding sequence of *APP exon 16* had been fused to APP$_{695}$-cDNA sequences up to amino acid 680, followed again by a stop codon. In both lines, *loxP sites* had been introduced before *exon 17* and in an intronic sequence after *exon 17*. In a second step, this sequence was deleted by transient transfection with a cre recombinase expression vector (Fig. 2.1 B), inactivating the APP region downstream of the inserted coding sequences. This led to two truncated forms of the APP protein, one only consisting of the

soluble, normally excreted ectodomain of APP and the other of a variant of APP that was still membrane-anchored, but lacked the intracellular interaction motif and thus a functional APP intracellular domain. So, both strains express the mutant APP transgenes on an APP null background, i.e. without expression of wildtype APP. In both cases, APPsα is still secreted into the extracellular space, however no activity-dependent regulation is possible in APPsα-expressing mice. Processing of the APPΔCT15 construct was normal, however the amounts of membrane-bound APP were larger, most likely because the lack of the interaction motif mediating endocytosis of APP led to reduced turnover. A detailed description can be found in the publication presenting the mutant mice (Ring et al., 2007).
Combined doubly gene-targeted mice, expressing either truncated version and lacking the *APLP2* gene, had been obtained crossing APLP2$^{-/-}$ mice to heterozygous APPsα or APPΔCT15 expressing mice, resulting in APLP2$^{-/-}$/APPsα and APLP2$^{-/-}$/APPΔCT15 expressing mutants and WT APP/APLP2$^{-/-}$ littermates.

Some of the experiments were performed on aged animals delivered only prior to examination, but most of the lines were bred in the central animal facility of the TU Braunschweig from mating pairs obtained from the laboratory of Prof. U. Müller. All animals were housed in groups in standard caging with unlimited access to food and water, light and dark periods being 12 hours each. Strains that were not bred, were allowed to get accustomed to the new environment for a minimum of two weeks before examination. These included the strains APLP2 knock-out, APPsα, APPΔCT15 and a sample of APP$^{-/-}$ mice.
For breeding, heterozygous mating pairs of two females and one male were housed in one cage. Usually, after weaning litters were marked with an ear clip and a tissue sample for determination of the genotype was collected under brief anaesthesia with diethylether. In experimental series on juvenile (p14-22) animals, the genotype was determined before weaning. Here, under anaesthesia, a mark with histology color (WAK Chemie, Germany) was applied under the skin of the palms for identification. In all cases, the genotype was verified after experimentation. Apart from inevitable cases, only littermates were used for experiments and measured whithin a narrow time window.

2.1.2 Genotyping

For DNA extraction for PCR analysis of the APP knock-out mouse line, tissue samples were lysed overnight in 100 µl "Gitschier" lysis buffer containing 100µg/ml Proteinase K at 55°C. The lysate was cleaned from cell debris by centrifugation and used 1:50 for PCR analysis.
For DNA-isolation for genotypisation of the knock-in lines, the tissue was lysed overnight in 500 µl standard lysis buffer containing 100 µg/ml Proteinase K at 55 °C. After cleaning the

lysate from cell debris by centrifugation, proteins were cleaned from the lysate before DNA was precipitated by mixing with an equal volume of phenol-chloroform (Roth, Germany). After centrifugation, the top phase was carefully collected and again centrifuged. The upper phase was again collected and mixed well with isopropanol. After a last centrifugation step the supernatant was discarded and the pellet washed with 70% ethanol. After complete air drying the DNA pellet was resuspended in 50 µl Tris-buffer for 1 hour at 37 °C before storage at – 20°C.

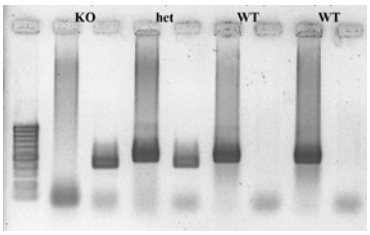
Afterwards, for detection of the wildtype or construct gene, the respective DNA was amplified by polymerase chain reaction (PCR) and detected after separation of PCR products on an electrophoresis gel. For APP detection a PCR kit (GoTaq® Polymerase, GoTaq® Flexi Buffer, $MgCl_2$ solution and nucleotide mix) by Promega (Wisconsin) and for detection of alpha and delta transgenes more sensitive Taq-Polymerase plus Polymerase buffer by GE Healthcare (illustra^{TM} Taq, GE Healthcare, Great Britain) were used. Primers were manufactured at Invitrogen (Invitrogen Corporation, USA). See table 2.3 for primer sequences, table 2.4 for PCR cycling mixes, table 2.5 for cycling protocols. Because of the small size difference of the PCR products, for detection of *APP*, wildtype and deletion mutant were amplified in separate PCR probes using APP For02 for WT and P3hygro for KO.

PCR products were separated electrically in an 1.5% agarose (Sigma) gel, detected after ethidium bromide (Sigma) staining under ultraviolet light and photographed (digital camera, Olympus). The images were edited with Adobe Photoshop (Adobe, USA). PCR products were found at basepair (bp) sizes indicated in table 2.2, representative gels are shown in figure 2.2.

Table 2.2 / Figure 2.2: PCR products. PCR gels with amplificates for WT and KO APP **(A)** and APPsα- and APPΔCT15 constructs and WT alleles **(B)**.

Primer Combination	Gene	Size of PCR product
APPfor02/UM42	Wildtype APP	476 bp
UM42/P3hygro	APP KO construct	410 bp
SR068/SR069	Wildtype APP	674 bp
SR068/SR071	APPsα/APPΔCT15 construct	308 / 508 bp

A



B

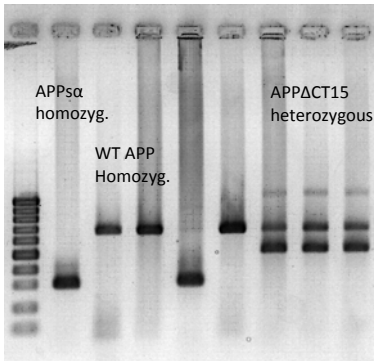


Table 2.3 Primer sequences for amplification of WT and deleted APP **(A)** and for APPsα- and APPΔCT15 constructs and WT APP **(B)**.

	Primer Code	Sequence
A	APPFor02	5´-ATGGTGGGCGTTGTCATAGCAA-3´
	UM42	5´-ATCACCTGGTTCTAATCAGAGGCCC-3´
	P3Hygro	5´-CGAGATCAGCAGCCTCTGTTCCACA-3´
B	SR068	5´- GGCTGACAAACATCAAGACGGAAGAG-3´
	SR069	5´- CACACCTCCCCCTGAACCTGAAAC´
	SR071	5´- CTGCGAGAGAGCATCCCTACAACC-3

Table 2.4 Composition of PCR mixes for amplification of WT and transgenic APP **(A)** and for APPsα- and APPΔCT15 constructs and WT **(B)**.

A	**Substance**	**Volume (µl)**
	DNA probe	1
	Polymerase buffer (5x)	5
	dNTPs	0.5
	Primer UM42 (5 µM) Primer APP For02 (5 µM) (WT) *or* Primer P3hygro (5 µM) (KO)	0.5 0.5
	Polymerase (5 units/µl)	0.25
	$MgCl_2$ (25mM)	3
	H_2O	14.25
	Total volume	25

B	**Substance**	**Volume (µl)**
	DNA probe	1
	Polymerase buffer 10x)	2.5
	dNTPs	0.5
	Primer SR068 (10 µM)	0.5
	Primer SR069 (10 µM)	0.5
	Primer SR071 (10 µM)	0.5
	Polymerase (5 units/µl)	0.15
	$MgCl_2$ (25mM)	0
	H_2O	19.35
	Total volume	25

Table 2.5 Thermal cycling protocols for amplification of WT and transgenic APP **(A)** and for APPsα- and APPΔCT15 constructs and WT **(B)**. After an initial denaturation step, denaturation, annealing and elongation were repeated consecutively for the number of cycles indicated.

	A			B		
Process	**Temp. (°C)**	**Time (min)**	**Number of cycles**	**Temp. (°C)**	**Time (min)**	**Number of cycles**
Denaturation	94	5	1	94	3	1
Denaturation	94	0.5	38	94	0.75	35
Annealing	66	1	38	58	0.75	35
Elongation	72	0.5	38	72	1	35
Final elong.	72	3	1	-	-	1

2.2 Preparation of hippocampal slices

For preparation of transverse hippocampal slices, mice were briefly anaesthetized with CO_2 and decapitated. The scull was opened and the brain rapidly removed and transferred into ice-cold carbogenated (95% O_2, 5% CO_2) artificial cerebrospinal fluid (ACSF). As the duration of this preparation step is crucial for survival of neurons, it was not to exceed 90 seconds. The tissue was then cooled down for 3 minutes to minimize oxidative stress.
After separation of the brain hemispheres, hippocampi were dissected consecutively by separating them from the surrounding tissue with two rounded spatulas, carefully avoiding bending or direct touching. First, the striatum was removed from the medial side of the brain, then one spatula was inserted under the *fimbria hippocampi* and the hippocampus separated from the cortex by cutting the subiculum. This step was performed in ice-cold ACSF in order to keep hippocampi chilled and to reduce their own weight.

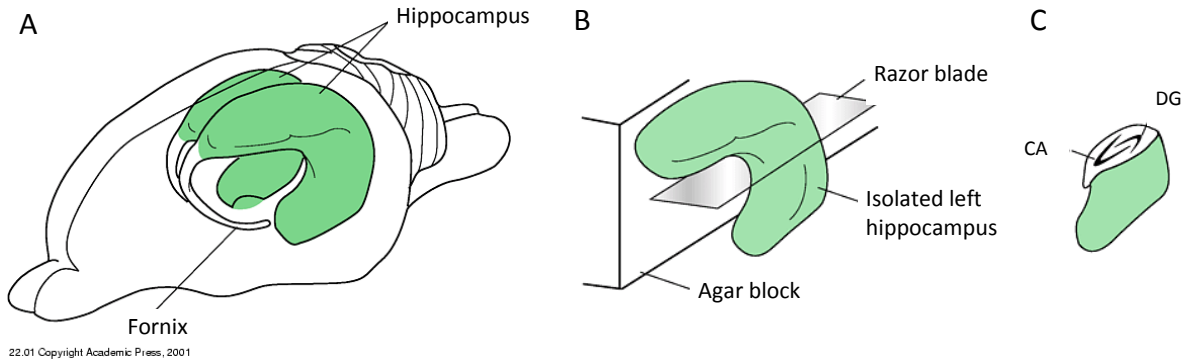


Figure 3.3 Localisation and preparation of hippocampi. **(A)** Localisation of the two hippocampi in a rat brain. Mouse hippocampi are slightly less curved and take up a slightly larger portion of the brain`s volume. **(B)** Preparated hippocampi are leaned against an agar block and sliced transversely. **(C)** This slicing leaves fiber connections between pyramidal cells intact. CA = cornu ammonis, DG = dentate gyrus. Adapted from Hammond (2003).

Hippocampi were then cut into slices of 400 µm thickness, transversly to their longitudinal axis (Fig. 2.3 B). In the first series of experiments on the $APP^{-/-}$ strain an egg-slicer (Katz, 1987) was used, in all other experiments a VT1000 vibratome (Leica Microsystems, Germany). The egg-slicer cuts tissue by letting a mesh of gold wire fall down onto the tissue in a guillotine-like fashion. This procedure is prone to damage the surface of the slices, as the gold wire has a comparably blunt surface, but is very quick, because within the fraction of a second all slices are cut. For vibratome slicing, both hippocampi were positioned and fixed upright on the specimen disk, leaning with the dentate gyrus onto an agar cube, then rapidly transferred into a buffer tray filled with ice-cold carbogenated ACSF. A vibrating blade cuts slice after slice by horizontal movements of the blade, alternating with upward movements of the specimen. This procedure causes less damage to the surface of slices, but is more time-consuming. In both

cases, during the cutting process damaged neurons fire at high frequency, which can trigger potentiation of synaptic connections. During vibratome cutting, this phase is prolonged to 5 minutes.
After preparation, slices were transferred into a "submerged" type storage chamber filled with carbogenated ACSF at room-temperature and allowed to recover for at least 90 minutes. If room-temperature exceeded 23°C, the chamber was placed above a styrofoam tray filled with crushed ice to maintain a temperature of approx. 19 – 21°C.

2.2.1 ACSFs

Slices were kept submerged in ACSF throughout preparation, storage and recording. In the process of optimisation of the preparation method, the composition of the ACSFs was adapted to the slicing procedure. In order to minimize firing of neurons during vibratome cutting, the ACSF used in combination with the egg-slicer ("standard ACSF") was changed in LTP experiments to different ACSFs for storage and recording ("vibratome ACSF"). Composition of the ACSFs see table 2.6.

Table 2.6 : Composition of the ACSFs used. All chemicals Sigma-Aldrich.

Substance [mM]	Standard ACSF	Vibratome ACSF for preparation and storage	Vibratome ACSF for recording
NaCl	124	125	125
KCl	3	2.5	2.5
KH_2PO_4	1.25	-	-
$MgSO_4$ * 7 H_2O	2	-	-
NaH_2PO_4 * H_2O	-	1.25	1.25
$MgCl_2$ * 6 H_2O	-	2	1
$NaHCO_3$	26	26	26
$CaCl_2$	2.5	2	2
D(+)-Glucose	10	26	26

Slicing with a vibrating blade is generally more gentle to the surface of the slice, resulting in larger numbers of cells and axonal and dendritic processes preserved. However, whereas cutting of all slices with the egg-slicer only takes the time the gold net takes to "fall" through the slice, during vibratome preparation the slicing procedure takes about 5 sec for each side of a slice.

To protect slices from potentiation during preparation, I changed ACSFs and also used different solutions for preparation and recording. For vibratome prepared slices I lowered the concentrations of K^+ and Ca^{2+}. For recording from those slices I additionally lowered the concentration of Mg^{2+} to facilitate release of the Mg^{2+}-block during LTP. Slices were let to acclimatize to the altered ional environment before start of recording. In order to adapt the slice to the conditions in the recording chamber, temperature and different ACSF, slices were pre-incubated in the chamber for 20 min.

2.3 Electrophysiology

Recording and data acquisition were performed on two setups comprising the same elements, yet partly from different manufacturers. In a set of preliminary experiments it was assured that experiments on both setups yielded comparable results and adaptations were made if necessary (level of liquid in the bath chamber, filter settings). Manufacturers' names refer to setup I and II, respectively.

For recording, slices were placed in a submerged-type recording chamber (Series 20, Warner Instruments, Conneticut or alternatively in a custom-made, MPI Martinsried) perfused with carbogenated ACSF at 1 ml/min via a minipulse pump (Ismatec, Switzerland / Abimed, Germany) and PVC Tubing (Braun, Germany and Ismatec / Abimed). The Warner chamber was mounted onto an aluminium platform (Warner Instruments) and the medium was heated by combination of a thermistor assembly inserted in the platform and an in-line solution heater (Warner Instruments), preheating the incoming ACSF. Both were controlled by a Dual Channel Heater Controller (TC-344B, Warner Instruments). The custom-made chamber consisted of a heatable steel body that also preheated the ACSF before reaching the chamber. Heating wires on the rear side of the chamber and a temperature probe sensing the temperature in the ACSF were coupled into a controlling device (DB-100, Mawitherm, Germany) that kept the temperature at constant values +/-0.2 °C. For recording of fiber volleys, the temperature in the recording chamber was kept at 22.5 - 23.5 °C, all other recordings were performed at 32°C.
ACSF was suctioned from the recording chamber by a steel cannula (Warner Instruments / Braun, Germany) connected to the tubing. To enable stable recordings, pulsation in the medium had to be kept to a minimum. This was ensured in both setups using larger tubing for suction than for influx of ACSF, combined with a position of the suction device that allowed simultaneous suction of medium and air.

Due to the lamellar organisation of the hippocampus, in a transverse slice preparation the majority of intra-hippocampal connections are preserved. Field excitatory postsynaptic

potentials (fEPSPs) in area CA1 were evoked by stimulation of the Schaffer Collaterals with an electrode placed in *stratum radiatum* of the CA3 region. The recording electrode was placed in the terminating region of the Schaffer Collaterals in the *stratum radiatum* of the CA1 region (Fig. 2.5). Recordings were started as soon as adaptation of the slice to the changed temperature and ACSF composition was observed by constant size of biosignals. Both electrodes were placed under visual control by a stereo microscope (Nikon, Japan / Wild Heerbrugg at Leica Microsystems, Germany).

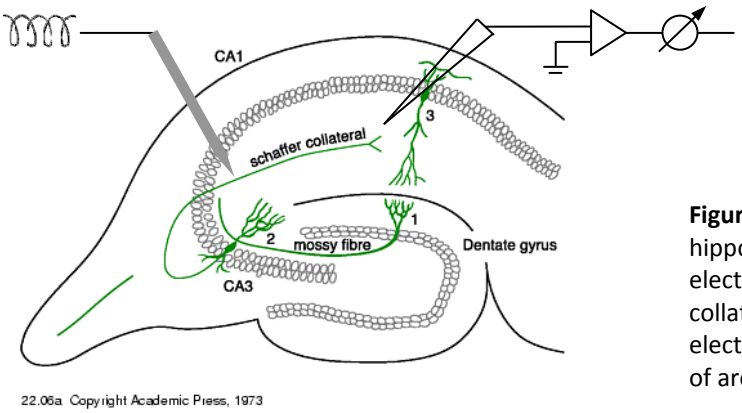


Figure 2.5 Placement of electrodes in hippocampal transverse slice: The stimulation electrode was positioned in the Schaffer collateral axons in area CA3, the recording electrode was inserted in in stratum radiatum of area CA1. Adapted from Hammond (2003).

2.3.1 Stimulation

Stimuli were delivered by 10 MΩ monopolar tungsten electrodes (WPI, USA). They were generated by a stimulus isolator (A360, WPI), connecting the negative pole to the electrode, the positive pole an Ag/AgCl-Pellet (WPI) that also served as indifferent electrode of the amplifyer´s headstage. Stimuli consisted of monophasic square pulses of 0.2 ms duration with stimulus sizes that ranged from 15 to 150 µA, depending on age of the animal and quality of the preparation. The stimulus pattern was generated by a master trigger (Master-8, A.M.P.I., Israel) synchronizing stimulation and data aquisition. The stimulation electrode was mounted on a mechanical micro-manipulator (Leitz, Germany).

2.3.2 Extracellular Recording

With a differential amplifier, potential alterations between two electrodes over time are measured. As in the indifferent electrode no potential changes occur, potential alterations reflect ional and electric changes in the vicinity of the recording electrode, placed in the tissue

of interest. Neural activity of single cells cannot be detected by this method, but in the hippocampal preparation, large unidirectional potential changes of populations of neurons occur simultaneously due to dense packing and unidirectional alignment of pyramidal neurons and can be recorded as summated potential changes. In the target region of the Schaffer Collaterals the depolarising influx of kations into dendrites creates an extracellular current sink, reflected as relative hyperpolarisation. An electrode localized in this region therefore records a negative potential change. With increasing intensity of stimulation, the number of activated synapses and therefore the amount of depolarisation increases.

Bioelectrical potential were recorded in the *stratum radiatum* of CA1 with borosilicate glass electrodes (1mm outer diameter, Biomedical Instruments, Germany). Electrodes were pulled from capillaries with a horizontal electrode puller (Flaming Brown P-97, Sutter Instruments, USA) to yield tip resistances of 10-20 MΩ when filled with 3M NaCl.
The pulled capillary was fitted either into an electrode holder, connected to the amplifyer´s headstage or directly into the electrode holder of the headstage (both Axoclamp-2B Axon Instruments at Molecular Devices, USA). These were mounted either on a mechanical (custom-made) or a computer programmable (Luigs & Neumann, Germany) micromanipulator. To exactly determine the depth of the electrode in the tissue, the electrode position at which the tip touched the surface of the tissue was determined by observing the transient alteration of the tip resistence of the electrode on an oscilloscope (HAMEG, Germany).
Recordings were started when a stable size of the signals indicated that the slices had acclimatized to the altered temperature and ional conditions of the medium in the recording chamber. For determination of the stimulus strength to be applied, the minimal stimulus strength that yielded a population spike in the fEPSP, was determined and stimulus strength was adjusted to elicit fEPSPs approx. 40% in LTP experiments, 30% for primed LTP and 60% for LTD induction.

In all measurements, the slope of the initial increase of the fEPSP was measured. In recordings of summated postsynaptic activity, depolarising EPSPs of excitatory synapses and hyperpolarising IPSPs (inhibitory postsynaptic potentials) of inhibitory synapses converging on the dendrites of pyramidal neurons, are included. IPSPs have slightly longer latencies, as they result from activation of interneurons by the Schaffer Collateral stimulation, mediating feed-forward inhibition and hence are disynaptic. Therefore they curtail the length of the fEPSP without decreasing the initial slope. The initial slope of the fEPSP is therefore a measure of the monosynaptic excitatory CA3-CA1 connection whereas the decay time of the depolarisation is shaped by inhibitory inputs.
The population spike is the summated axonal signal of CA1 pyramidal neurons, overlaying the initial slope of the fEPSP and was avoided because it does not reflect the strength of the synaptic activation of CA1 neurons, but the strength of the summated axonal output of CA1

pyramidal neurons. Its polarity is contrary to the fEPSP, because here, the dendritic region serves as current source to compensate for the strong current sink generated in the *stratum pyramidale*. Figure 2.6 shows how the population spike component contaminates slope and amplitude of the dendritic signal.

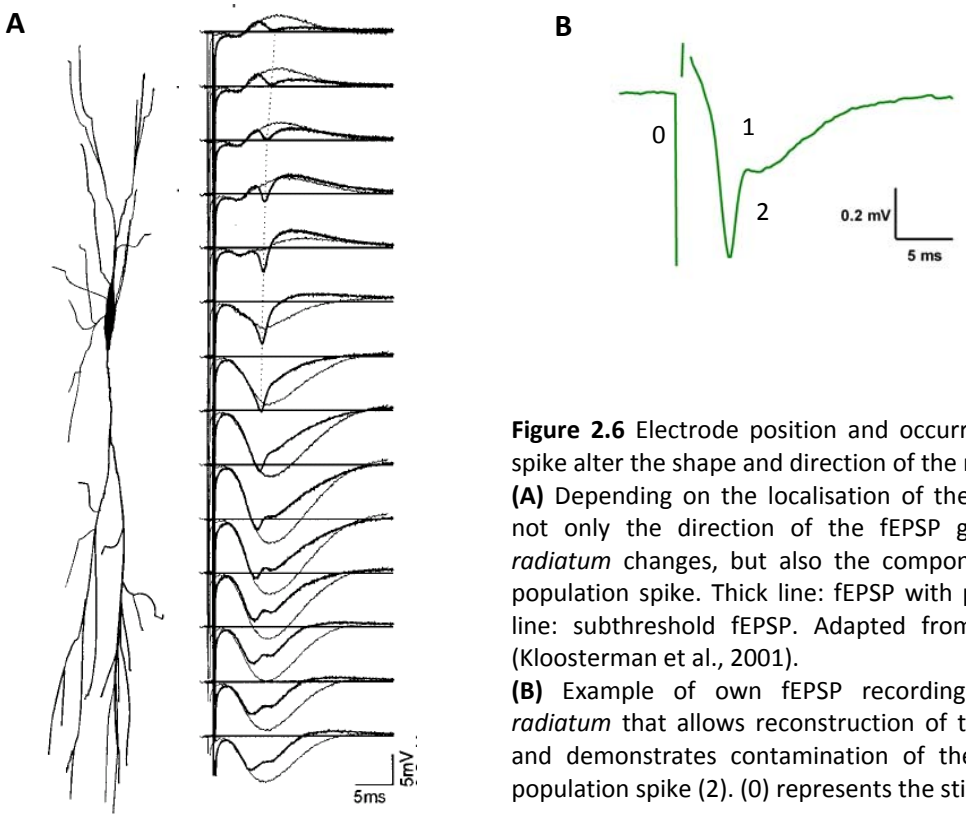


Figure 2.6 Electrode position and occurrence of a population spike alter the shape and direction of the recorded fEPSP.
(A) Depending on the localisation of the recording electrode, not only the direction of the fEPSP generated in *stratum radiatum* changes, but also the component representing the population spike. Thick line: fEPSP with population spike, thin line: subthreshold fEPSP. Adapted from Kloosterman et al. (Kloosterman et al., 2001).
(B) Example of own fEPSP recording from CA1 *stratum radiatum* that allows reconstruction of the electrode position and demonstrates contamination of the fEPSP (1) with the population spike (2). (0) represents the stimulus artefact.

2.3.3 Stimulation protocols

I used a selection of stimulation protocols to examine basal synaptic properties, short-term plasticity and different forms of long-term plasticity. In all cases I referred to the slope of the fEPSP. An overview over the protocols is given in Table 2.6 (next page).
Basal synaptic transmission was evaluated by determining the size of fEPSP responses either to a range of stimuli of the Schaffer collaterals of different sizes or to fiber volley amplitudes. The fiber volley (Fig. 2.7, next page) represents the summated signal of stimulated Schaffer Collateral axons and therefore allows a more direct correlation of stimulation strength and fEPSP response. It was not measured at 32°C, but at room-temperature, as then EPSP latencies are longer and do not overlap the fiber volley.

Table 2.6 Stimulation protocols used in this work.

Stimulus	Protocol
EPSP size measurement and "baseline" recording	single pulses at 0.1 Hz
Paired pulse facilitation	pulse pairs with inter-pulse frequencies of 160, 80, 40, 20, 10 ms; repeated at 0.1 Hz
Tetanus	30 pulses at 100Hz, repeated 3x
Theta burst stimulation (TBS)	10 bursts of 4 pulses (50/100Hz), separated by 200 µs, repeated 3x
Primed LTP	1 100Hz TBS, followed 30 min later by regular 3x 100Hz TBS
Single-pulse low frequency stimulus	900 single pulses at 1 Hz
Paired-pulse low frequency stimulus	900 pulse pairs at 1 Hz, inter-pulse interval 50 ms

Presynapse function and short-term plasticity were investigated by determining the ratio of the size of responses of two closely spaced stimuli, using inter-stimulus intervals of 160, 80, 40 20 and 10 ms. When two stimuli are presented in a short inter-pulse interval, the synaptic release of transmitter is modulated in a predictable fashion at a given synapse type (Stevens and Wang, 1995; Hjelmstad et al., 1997). The gain in synaptic transmission from the first to the second pulse is then evaluated as paired-pulse facilitation (PPF), which, expressed as percentage, is equal to (fEPSP2 / fEPSP1)*100.

To investigate activity-dependent synaptic plasticity, I chose four protocols for long-term potentiation, i.e. permanent enhancement of the synaptic response (Tetanus, theta burst stimulation with two frequencies and primed LTP), and two for long-term depression (stimulation with single pulses and pulse pairs), resulting in permanent decrease of fEPSPs. I recorded fEPSPs for 20 minutes at a frequency of 0.1 Hz, then induced LTP or LTD and maintained recording at baseline frequency for another 60 minutes or 200 minutes for late LTP. The ratio of potentiation or depression was calculated as % of fEPSP slope value compared to the average value before induction.

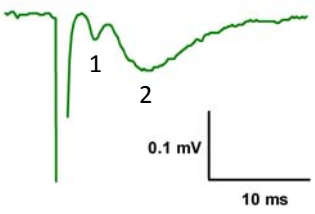


Figure 2.7 Example of a signal recorded in CA1 at room-temperature. (1) fiber volley, (2) fEPSP.

LTP is induced by high-frequency stimulation. The simplest protocol is a tetanus, consisting of a long burst of high-frequency pulses. I applied bursts consisting of 30 pulses at 100Hz, repeated 3 times at 0.2 Hz.
The theta burst stimulation protocol consists of short bursts with intermittent pauses. I applied trains of 10 bursts, consisting of 4 pulses at 50 or 100Hz with 200 µs intervals between bursts. In total, 3 such stimulus trains were given at 0.2 Hz. This stimulus pattern is regarded as more physiological as it closely resembles the periodicity of the theta-rhythm, a pattern of network activity that is predominant in the hippocampus during movement and exploration (Sainsbury et al., 1987).
For primed LTP, one theta burst train was applied after baseline recording, followed by the regular 3 theta bursts after 30 more minutes. The state of the synapses before the second pulse is different from before the first pulse, because the recent prior activation modulates excitability and reactivity to further stimuli by not yet fully clarified mechanisms. (Christie et al., 1995; Christie and Cameron, 2006; Mellentin et al., 2007).
LTD is induced by prolonged low-frequency stimulation. There are different forms of LTD, distinguished by the intracellular processes they trigger and that are induced by differences in the protocol. I used either 900 single stimuli at 1 Hz or 900 pulse pairs at 1 Hz, and with 50 ms intervals within the pulse pairs.

2.3.4 Pharmaca

When regular ACSF was replaced by ACSF containing pharmaca, the necessary volume of solution was prepared freshly for each experiment. Beforehand, I had verified that maintenance of pH and oxygenation level in the volumes used (35 and 65 ml) did not change or negatively affect slice viability or LTP induction.
Picrotoxine (PTX), a non-competititive antagonist at the $GABA_A$ receptor, was used in 20 µM concentration in experiments on aged $APP^{-/-}$ mice. The PTX stock solution was prepared by dissolving PTX (Sigma-Aldrich, USA) in DMSO (Sigma-Aldrich). In preliminary experiments comparing LTP induction rates with and without DMSO, I excluded that DMSO, at a final concentration of 0.5‰ in the ACSF, had an effect on LTP induction rates. PTX was applied after 10 minutes of baseline until the end of the experiments. The experiment was not continued if the baseline rose more than 10% under the influence of PTX.
APV, a selective competitive antagonist at the NMDA receptor, was used in LTD experiments and applied at a concentration of 50 µM from 10 minutes before low-frequency stimulation until 5 minutes after. The APV stock was prepared in ddH_2O.

2.4 Data acquisition

Recordings were performed with an Axoclamp2B differential amplifier (Axon Instruments at Molecular Devices, Sunnyvale, California) in bridge mode. Signals were amplified 200x and bandpass-filtered to a bandwidth of 1 to 1700 Hz (LHBF-48x-4HL NPI, Germany) and 1 to 1000 Hz (custom-made, electronic department, MPI Martinsried), respectively. Filter settings were adapted to yield biosignals of identical size and slope at both setups by feeding the signal from one amplifier into both downstream hardware components.
Amplified and filtered signals were sampled at 5 kHz and fed into a PC via multi-IO card (National Instruments, USA). Data aquisition and analysis were performed with a custom-made program (Ana-DAP, M.Korte, V.Staiger), written with National Instruments CVI Software (National Instruments, USA).

2.5 Data analysis

Online and subsequent offline re-analysis of raw data were also performed with NI-based software by M. Korte and V. Staiger. The initial slope of the fEPSP was calculated within a set time window as the slope of a fitted curve. For PPF data analysis, the off-line analysis program ANA PPF additionally divided the slope value of the second pulse by the slope value of the first pulse. Pre-processed data were saved as Microsoft Excel (Microsoft, USA) files.
Further offline analysis was performed with Microsoft Excel. For calculation of fEPSP sizes in response to stimulation and for PPF ratios, three values for each parameter (stimulus strength, fiber volley amplitude or inter-stimulus interval) were averaged. The ratio of paired pulse facilitation (PPF) was calculated as (EPSP2 slope/EPSP1 slope)*100. In experiments on long-term potentiation and long-term depression, the values of six sweeps, corresponding to the responses during one minute, were averaged. These values were normalized to the mean fEPSP slope value before induction of potentiation or depression, constituting the baseline and calculated as (EPSP slope/ mean EPSP$slope_{baseline}$)*100.
Experiments were excluded from the analysis, if the values during baseline acquisition exceeded 20% increase or decrease in signal size, as this trend would have contaminated the course of the potentiation levels during the following hour. For the same reasons, LTD experiments were discarded if the gradient of regression line of maintenance exceeded -5% between minute 43-48 and minute 93-98.

Statistical analysis was performed with Microsoft Excel (Microsoft, USA) and GraphPadPrism version 5.00 for Windows, (GraphPad Software, USA). Data are presented as means with error bars indicating standard error of the mean (SEM).

Gaussian distribution of data was determined by D`Agostino-Pearson test, allowing subsequent use of student´s t-test. Significances were determined by two-tailed student´s t-test, applied to the mean values for each inter-stimulus interval in PPF experiments and to mean values 55-60 minutes after LTP induction in LTP experiments or 45-50 and 85-90 minutes in primed LTP experiments, accepting $P < 0.05$ as significant and $P < 0.01$ as very significant. For progression of LTD induction rates with age, a regression line was fitted through the mean values of depression after 60 minutes. The linearity of the regression was confirmed by runs test. A possible difference between regression lines of the different genotypes was determined by 95% confidence intervals.
All graphs were generated with GraphPadPrism.

2.6 Histology

Hippocampal slices from electrophysiological experiments were fixed overnight in PFA (Sigma, 4% w/v in phosphate buffer) and dehydrated in 30% sucrose (Sigma, 4% w/v in phosphate buffer) for cryoprotection. The tissue was cut into 30 µm slices with a freezing microtome (Reichert, Austria) and mounted on gelatine-coated microscope slides.
For assessment of pyramidal cell numbers in CA3 and CA1 region, anterior hippocampal slices were used. After thorough drying at room temperature, slices were incubated for 2 minutes in DAPI (4'6-di-amidino-2-phenylindol) solution (1:1000 in phosphate buffered saline, PBS) to detect nuclei, rinsed extensively with PBS and preserved in anti-fading mounting medium (Fluoro-Gel, Science Services, Germany).
For immunohistological stainings slices were permeabilized and pre-blocked with 2% NGS and 0.2% Triton X-100 in PBS) for 1h at room-temperature and incubated overnight at 4°C with anti-GFAP primary antibody (Sigma G6171, 1:400 in blocking solution). After rinsing, primary antibody was visualized on slices with Cy^{TM}2-conjugated secondary antibody (1:500 in PBS, Dianova, Germany) and slices rinsed extensively before mounting.
Slices were viewed at a microscope (Axioplan 2, Zeiss, Germany) equipped with epifluorescence illumination at 2.5x and 10x magnification and photographed with a digital camera (AxiocamMR, Zeiss, Germany). Pictures were processed with Adobe Photoshop CS (Adobe, USA). In DAPI stained slices a 500 x 300 mm region in CA3 and CA1 was determined for counting. Counting of DAPI stained nuclei within the stratum pyramidale of CA3 and CA1 was done with ImageJ v.1.37 freeware (NIH, USA) with cell counter plugin. Values were analysed with Excel and GraphPad Prism (GraphPad Software, USA).

3 Results

In my work I assessed the role of APP and its homolog APLP2 in synaptic function and plasticity, using *in vitro* electrophysiological methods. My focus of interest lay on the functions of the parts and active cleavage products of both proteins in the intact organism. In a loss of function approach, I examined several lines of gene-targeted mouse mutants carrying deletion mutations for either holo-APP or parts of the protein, in some combined with deletions of APLP2. As I wanted to examine whether and how APP is involved in synaptic functions related to processes of learning and memory, I focussed my studies on the hippocampus as the brain region responsible for learning and memory function and as the structure that is affected first in Alzheimer´s Disease. Here I investigated the functionality of the CA3-CA1 synapse, the model synapse for assessment of the functionality of information processing in the hippocampus and for memory-related tasks.
I started my experiments on mouse strains carrying single mutations leading to truncations of the APP protein and later extended them onto mice carrying additional deletions of the APP homologue APLP2.

3.1 Investigation of the role of APP in synaptic plasticity

My first aim was to determine whether aged mice without a functional *APP* gene (APP knock-out or $APP^{-/-}$) have deficits in long-term potentiation (LTP) and synaptic transmission. If so, I wanted to identify the responsible region of the APP protein by extending my experiments onto mice expressing different truncated versions of APP. Experiments were performed directly after behavioral testing for learning and memory deficits in the group of Prof. D. Wolfer, ETH Zürich. Next, I specified involvement of inhibitory synaptic activity to the observed alterations.

3.1.1 Aged APP knock-out mice

To investigate synaptic plasticity, Martin Korte and I induced long-term potentiation (LTP) at the hippocampal CA3-CA1 synapse of aged $APP^{-/-}$ and WT littermates. In acute hippocampal slices, we stimulated the Schaffer Collaterals, axons of CA3 pyramidal neurons

connecting to neurons in the CA1 region and recorded extracellular field EPSPs (fEPSPs) in the apical dendritic region of CA1 neurons. Stimulus strength was adjusted to produce an fEPSP slope value 40% of that at which a population spike was observed first. After 20 minutes of baseline recording at a stimulus frequency of 0.1 Hz, theta burst stimulation (TBS), consisting of 10 trains of 4 pulses at 100Hz with an inter-burst interval of 200 μs, repeated 3x, was applied and recording was continued for further 60 minutes.
We found that deletion of the APP gene resulted in significantly reduced rates of both induction and maintenance of LTP in aged $APP^{-/-}$ mice without parallel neurodegeneration (Fig. 3.1 A, next page). Values before and after LTP induction are expressed in % of the mean fEPSP size before LTP induction (baseline), calculated as (fEPSP / mean $fEPSP_{baseline}$)*100. However, LTP was not abolished completely, reaching slightly less than half of the potentiation values of wildtype littermates. 60 minutes after TBS, slices from $APP^{-/-}$ mice displayed 142.9 %, wild-types 212.0% potentiation. This was paralleled by reduced performance in memory-related behavioral tasks (Ring et al., 2007). Unimpaired LTP-induction in juvenile $APP^{-/-}$ animals excluded that this defect was present age-independently (Fig. 3.1 F).

I also assessed paired-pulse facilitation (PPF) and basal synaptic transmission, expressed as the strength of responses to excitatory synaptic stimulation. When axons are stimulated with a short inter-pulse interval, the synaptic release of transmitter in active synapses can be modulated in a predictable fashion (Stevens and Wang, 1995). PPF characterizes the increase of synaptic response to the second of two successive pulses within the range of milliseconds. It is a form of short-term plasticity, because its effects are transient and is regarded as a measure to assess the functionality of the presynapse, because it is predominantly governed by presynaptic mechanisms modulating transmitter release probability. The gain in synaptic transmission from the first to the second pulse of EPSP slope is expressed in % of the size of the first EPSP and equal to ($fEPSP_2$ / $fEPSP_1$)*100. In old $APP^{-/-}$ mice PPF was unchanged compared to WT littermates, excluding a consequence of the lack of APP on presynapse functionality.
fEPSPs sizes in response to stimuli of given strengths were identical in $APP^{-/-}$ and wild-type (WT) littermates (Fig. 3.1 D). The unaltered fEPSP sizes in aged $APP^{-/-}$ mice go in line with the absence of histopathological alterations, as determined by the synapse marker synaptophysin (Ring et al., 2007), the astrocytic marker GFAP (glial fibrillary acidic protein, Fig. 3.1 G) and by unaltered numbers of pyramidal cells in CA3 and CA1 (Fig 3.1 C). As this method of measurement is not independent of the number of axons stimulated, a more accurate measure is assessment of the size of the postsynaptic response in relation to the summated axonal signal from the Schaffer Collaterals (Fig 3.1 E). The so-called fiber volley reflects the amount of stimulated axons. The results from these experiments confirm the findings from fEPSP size assessment relative to strength of stimulation, however in some of the data sets, the number of experiments was too small to assess significances.

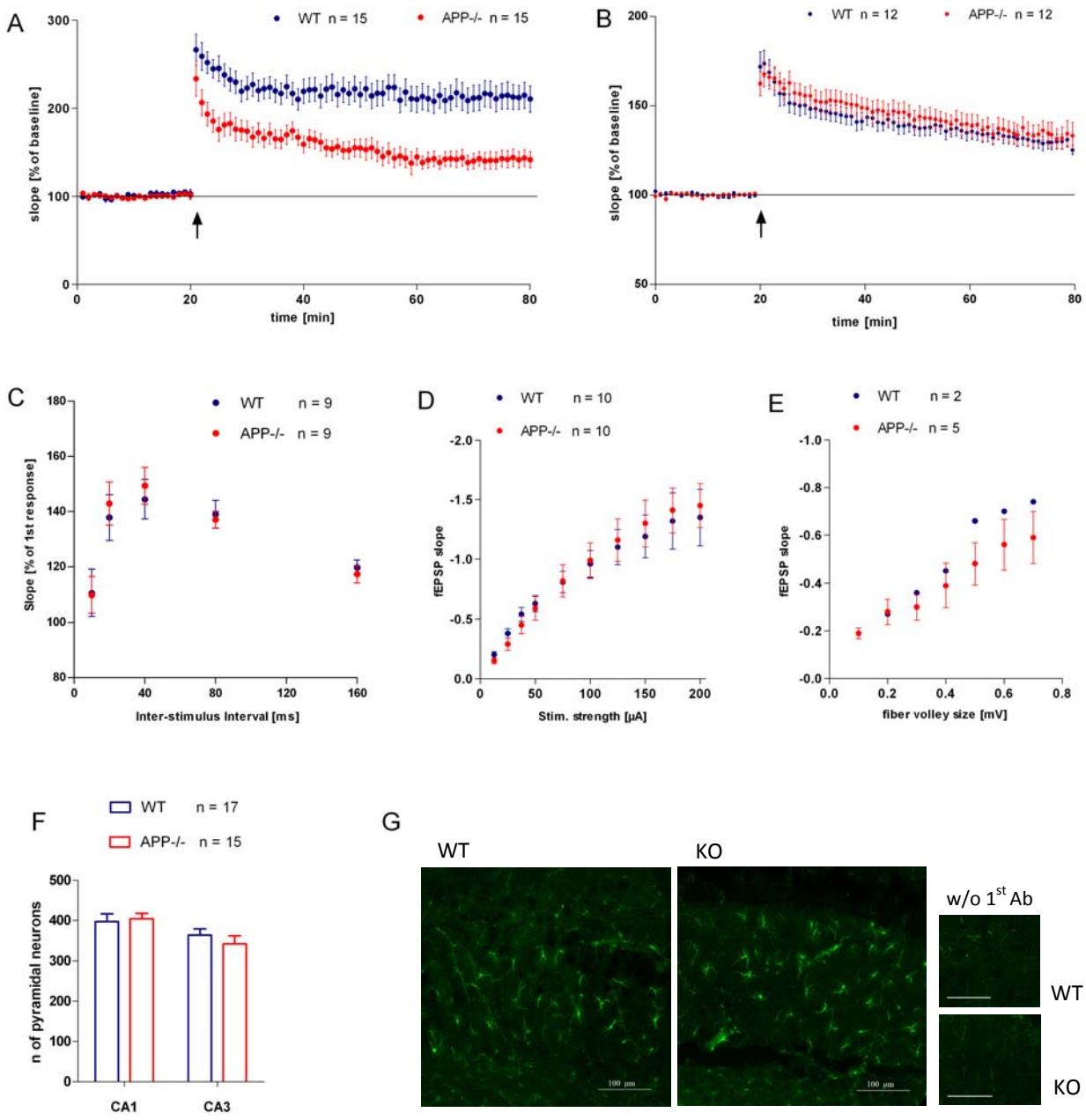


Figure 3.1: Deletion of the *APP* gene resulted in an LTP-deficit and smaller fEPSPs, but no alteration in paired pulse facilitation in aged (9-12 months) $APP^{-/-}$ mice. **(A)** $APP^{-/-}$ animals showed significantly lower rates of LTP induction and maintenance 60 min after TBS (arrow) (WT 212±14,5; APP 142,8±9,5; p = 0.0013, student´s t-test). Representative fEPSPs are shown for both experiments before and, overlayed, 60 min after TBS. **(B)** Juvenile (p14-p21) $APP^{-/-}$ animals did not have a deficit in LTP. **(C)** Presynapse functionality, assessed by paired-pulse stimulation with inter-stimulus intervals of 160, 80, 40 20 and 10 ms and expressed as gain of fEPSP slope in %, also did not differ in aged $APP^{-/-}$ and WT animals, as mirrored in unaltered facilitation rates. Basal synaptic transmission, assessed in relation to stimulus size **(D)** and in relation to the amount of axonal stimulation **(E),** was also unaltered in aged $APP^{-/-}$ mice. **(F)** Pyramidal cell numbers in CA1 and CA3 region were also identical. **(G)** Immunohistological staining for GFAP revealed no gliosis in aged $APP^{-/-}$ mice. All data are presented as means with error bars indicating the standard error of the mean.

These findings showed that APP exerts its effects on long-term potentiation not only via Aβ, but it is itself necessary for intact long-term potentiation throughout an animal´s life. The underlying mechanism could be an effect on the "machinery" for LTP, caused by lack of other parts of APP or an indirect result of anatomical or functional changes in the hippocampus, e.g. impaired inhibitory synaptic transmission.

3.1.2 Aged APPsα and APPΔCT15 expressing mice

Having found an age-dependent selective deficit in synaptic plasticity in $APP^{-/-}$ mice, I set out to determine the mechanism by determining the part of APP that is necessary for intact LTP. I repeated experiments on long- and short-term plasticity and synaptic transmission on two strains of aged gene-targeted mice from the group of U. Müller, IPMB Heidelberg, in which WT APP had been replaced by APP constructs corresponding either to the secretable APP ectodomain as generated by alpha-secretase cleavage ("APPsα") or to a C-terminal truncation that lacks the last 15 amino acids harbouring the intracellular YENPTY interaction and signaling domain ("APPΔCT15"). The genetic background of these mice was identical to that of the $APP^{-/-}$ mouse line.

Interestingly, LTP rates were unaltered in APPsα knock-in (KI) mice (Fig. 3.2 A, next page), although most of the putative functions of APP - as membrane-bound molecule as well as by activity-dependent cleavage - were excluded. The same was true for presynapse functionality and short-term plasticity, which was assessed by paired-pulse facilitation and basal excitatory synaptic transmission in general, correlating with unaltered numbers of pyramidal neurons in CA3 and CA1. This strongly hints to a central role of APPsα for maintaining intact activity-dependent plasticity in age.

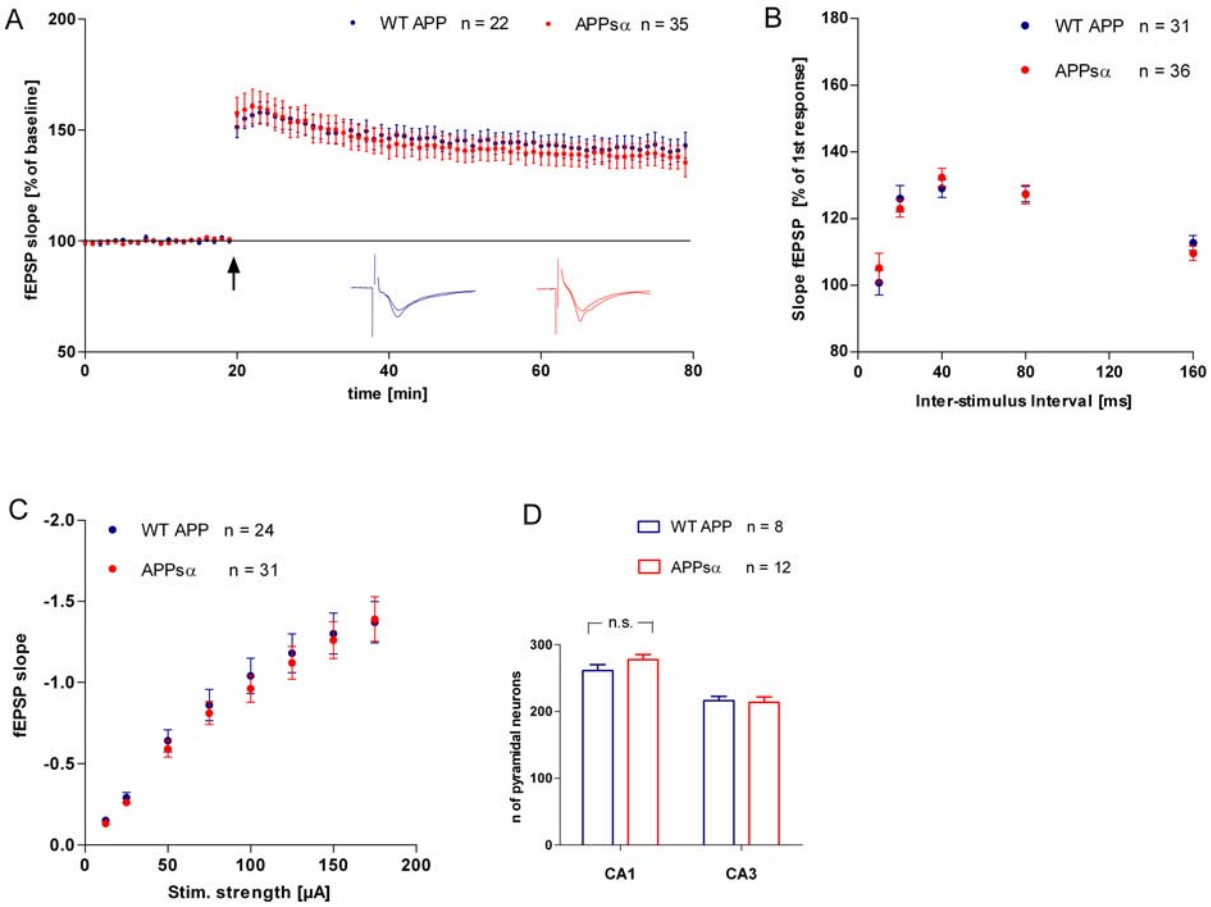


Figure 3.2 The deficits of aged APP$^{-/-}$ mice were rescued in APPsα knock-in mice. **(A)** LTP of fEPSPs was induced again by TBS (arrow). Unlike APP$^{-/-}$ mice, aged (12 - 15 months) APPsα expressing mice did not have LTP-deficits relative to their littermates expressing WT APP after 60 min (WT APP 142.0 ± 5.25, n = 22, APPsα 136.6 ± 6.96, n = 35), representative fEPSPs are shown before and, overlayed, 60 min after TBS. **(B)** Paired-pulse facilitation (PPF) as a measure of presynapse function and short-term plasticity, expressed as gain of fEPSP slope in % was also unaffected. Basal synaptic transmission **(C)** and the number of pyramidal neurons in CA1 and CA3 **(D)** were also not significantly altered. Error bars: standard error of the mean.

LTP in mice lacking the intracellular C-terminal YENPTY motif also was apparently unaffected (Fig 3.3 A, next page). Paired-pulse facilitation, basal synaptic transmission and pyramidal cell numbers were unaltered likewise (Fig. 4.3 B-D). Here, it is interesting to keep in mind the unaltered rates of paired-pulse facilitation for the outcome of later experiments on combined KO strains.

Induction rates in both sets of experiments were lower than in the experiments on APP knock-out mice. This can be attributed to several, possibly interacting, factors. Firstly, animals were about 3 months older than in the APP$^{-/-}$ series. Also, in spite of back-crossing to C57/Bl6 background, it is not unusual for different mouse strains to have greatly varying LTP induction rates. Therefore, the necessary and sufficient criterion is comparison of mutants to internal WT littermates as the closest possible control. Part of the effect, yet, was due to a change in

preparation method. Whereas slices of the APP$^{-/-}$ mice were produced with the egg-slicer method, for APP KI experiments I changed to preparation of slices with a Leica vibratome, as this slicing is more gentle for the tissue. Stabile baselines and low error bars show that vibratome-prepared slices were in good condition.

The necessary factor for the possibility to draw conclusions from data sets is the possibility to statistically distinguish between two groups of data. In the case of the APPΔCT15 mice, which had the lowest induction rates, error bars reached 6.45% of the potentiation after 60 minutes. So, a difference of 13% between induction rates would have been enough to produce no overlap of error bars and an induction rate of not more than 116% for the mutant animals would have produced a significant ($p < 0.05$) difference in student´s t-test.

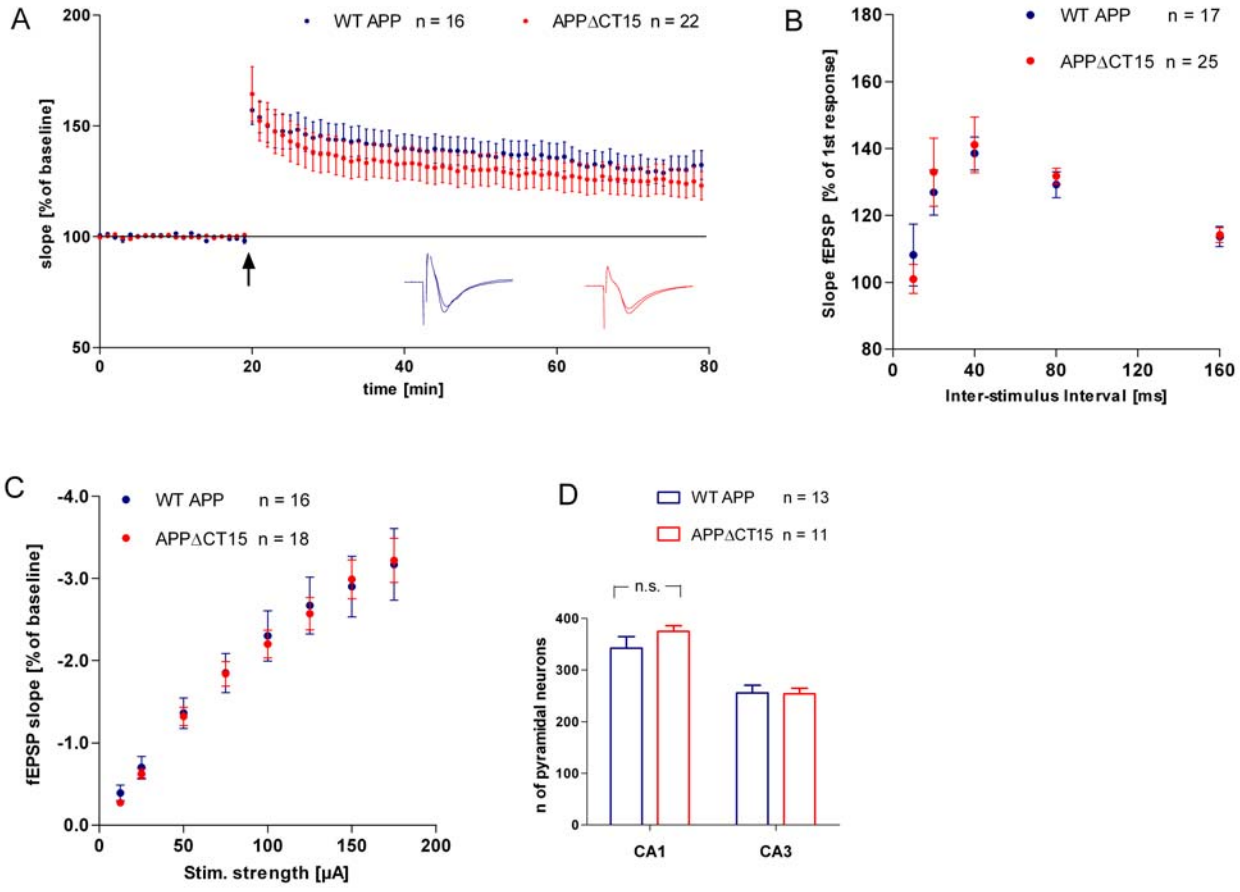


Figure 3.3 APPΔCT15 knock-in mice also displayed no LTP-deficit in old age. **(A)** Aged (12 - 15 months) APPΔCT15 expressing mice did not have LTP-deficits relative to their WT APP expressing littermates after 60 min (WT APP 131.9 ± 6.07; APPΔCT15 124.9 ± 6.45). Neither basal synaptic transmission **(B)** nor paired-pulse facilitation (PPF) **(C)** were altered either. Error bars: standard error of the mean.

In order to determine an additional role of the APP intracellular domain (AICD) on transcription-dependent late phases of LTP, I performed long-term experiments on aged APPsα-KI and APPΔCT15-KI mice. As the AICD is lacking in both mouse strains, I pooled data

from experiments of both strains. After 200 minutes, induction rates were with 115.9% and 115.7% not significantly different (Fig. 3.4, next page).
Although the stability of the fEPSP sizes over time is not satisfactory, owed to the fact that experiments were performed on average 12 h after preparation, the temporal course of LTP maintenance does at least not reveal severe deficits in transcription-dependent mechanisms, as e.g. apparent in BDNF$^{-/-}$ mice, which display an articulate drop of LTP back to 100% 120 minutes after induction (Korte et al., 1998).

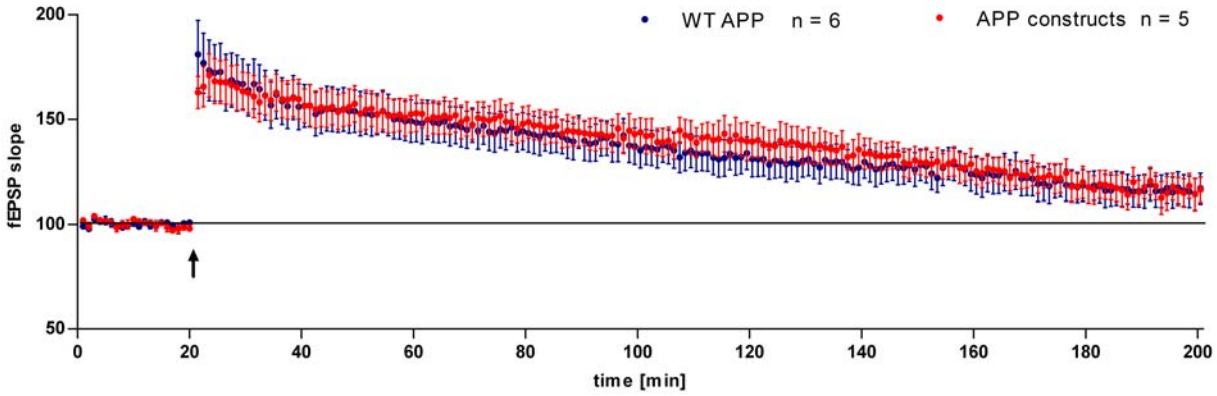


Figure 3.4 Also, the late form of LTP was not impaired in APPsα and APPΔCT15 expressing mice (WT APP 116.0 ± 7,43; APP construct 115.7 ± 6.57). Data from both strains are pooled.

So, I had been able to show that the deficits caused by the lack of APP were attributable exclusively to the missing of the α-secretase generated fragment of APP, APPsα, even presumably in forms of LTP that require gene transcription. This paralleled the findings from behavioral experiments, detecting learning deficits in a water maze place navigation task only in aged APP$^{-/-}$ mice, but not APPsα- and APPΔCT15-KI mice (Ring et al., 2007).
A remarkable finding was that the lack of the APP ectodomain on behavior and LTP only became effective in aged mice. Possible reasons were that APLP2, an APP homolog with almost identical sequence, largely overlapping expression pattern and similar processing, compensated for the lack of APP during most of the animal´s lifetime or that mechanisms were effective that only yield consequences in late stages of the animal´s life.

3.1.3 Blockade of GABAergic activity in aged APP knock-out mice

There were indications that the LTP deficit in old APP KO mice might be related to age-dependent alterations in the function of inhibitory neurons and their contributions to signal transmission in the hippocampus. The inhibitory neurotransmitter GABA (γ-aminobutyric

acid) is a key signaling factor controlling LTP (Wigstrom and Gustafsson, 1983; Davies et al., 1991). In another strain of $APP^{-/-}$ mice Seabrook et al. found attenuated paired-pulse depression of GABA-mediated inhibitory post-synaptic currents, that contributed to a mild LTP deficit (Seabrook et al., 1999). Also, decreased levels of GABA and loss of GABAergic neurons have been found in AD patients (Lanctot et al., 2004; Lanctot et al., 2007) in rodent models of AD (Ramos et al., 2006) and aging (Vela et al., 2003).

In order to test for inhibitory GABAergic contributions to the potentiation in aged $APP^{-/-}$ and WT mice of the strain used here, I induced LTP by theta burst stimulation under pharmacological blockade of $GABA_A$ receptor activation by picrotoxine, dissolved at 20 µM DMSO in the ACSF. In preliminary experiments comparing LTP induction rates with and without DMSO, I had excluded that DMSO at the concentration used (0.5‰) had an effect on LTP induction rates. Under these conditions I found the LTP deficit between WT and KO to be abolished (Fig. 3.5, next page).
Theta burst stimulation involves activity of both $GABA_A$ and $GABA_B$ receptors. Picrotoxine is an antagonist at the $GABA_A$ receptor. So, loss of $GABA_A$-mediated fast inhibition abolished the difference between $APP^{-/-}$ and WT animals. This leads to the conclusion that in aged $APP^{-/-}$ mice GABAergic neurotransmission via the $GABA_A$ receptor was affected and GABAergic contributions to long-term potentiation in aged $APP^{-/-}$ mice were altered.

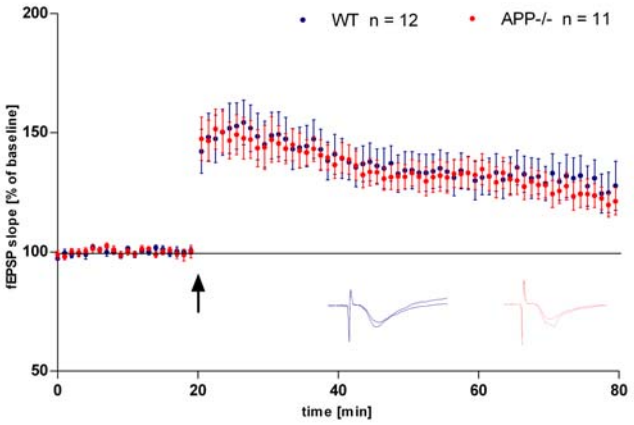


Figure 3.5 Inhibition of GABAergic transmission. Application of 20 µmolar concentration of the $GABA_A$-antagonist picrotoxine abolished the LTP difference between aged WT and $APP^{-/-}$ animals (WT 126.1 ± 8.69; $APP^{-/-}$ 122.1 ± 5.32). Representative fEPSPs before and 60 min after TBS show that picrotoxine did not cause severe distortion of the signal.

3.2 Specification of the role of APLP2 in long-term potentiation

The occurrence of LTP deficits only in old $APP^{-/-}$ mice suggested the question whether more severe deficits were masked by functional redundancy of the two proteins. The existence of overlapping functions is apparent, because combined KO animals for both APP and APLP2 are not viable and die shortly after birth, whereas $APLP2^{-/-}$ mice are viable and have no

obvious defects (von Koch et al., 1997; Heber et al., 2000). Also, both proteins are expressed in the whole organism and are evolutionarily highly conserved, indicating a vital function.
To specify the role of APLP2, I examined knock-out mice only for APLP2 and mice with combined deletions. Two further mouse strains generated in the lab of Prof. U. Müller expressed the same truncated versions of APP as the mice in the previous set of experiments, but had been crossed with APLP2$^{-/-}$ mice.

3.2.1 Aged APLP2 knock-out mice

To start, I investigated the contributions of APLP2 alone to intact synaptic plasticity and synaptic function. In APLP2$^{-/-}$ mice of the same age as the before tested APP$^{-/-}$ animals, no deficits were detected (Fig. 3.6 A, next page). 60 minutes after LTP-induction, WT and littermates reached potentiation levels of 164.9 and 161.9% respectively. Input-output relations and PPF were also unaltered (B-D). Behavioral testing of the same individuals immediately before LTP experiments had not yielded any differences either (D.Wolfer, personal communication).
In this and all following series LTP induction rates were higher than in the series of mice expressing the truncated APP constructs, but intact APLP2. Apart from the aforementioned differences between strains, the difference was due to adaptation of the ACSF in the process of establishing the preparation method with the Leica vibratome. The fact that not only induction rates had been fairly low, but also baseline EPSPs had been larger in slices prepared with the vibratome in combination with standard ACSF, suggest that part of synapses might already have been potentiated in the process of the preparation, leaving less ability to further potentiation until saturation. Damaging of neurons in the slicing process causes their membrane potential to break down which results in prolonged bursting activity which would resemble strong tetanic stimulation and cause subsequent potentiation of the CA3-CA1 synapse. To exclude the risk of potentiation during preparation, I changed ACSFs for preparation and recording, reducing the concentration of K^+ and Ca^{2+}. For recording I additionally reduced the concentration of Mg^{2+} to 1 mM in order to facilitate release of the Mg^{2+}-block during LTP induction. Such potentiation could most likely not occur when using the egg-slicer, as here cutting of all slices takes only a fraction of a second, whereas vibratome slicing takes approximately 5 seconds for each side of a slice.

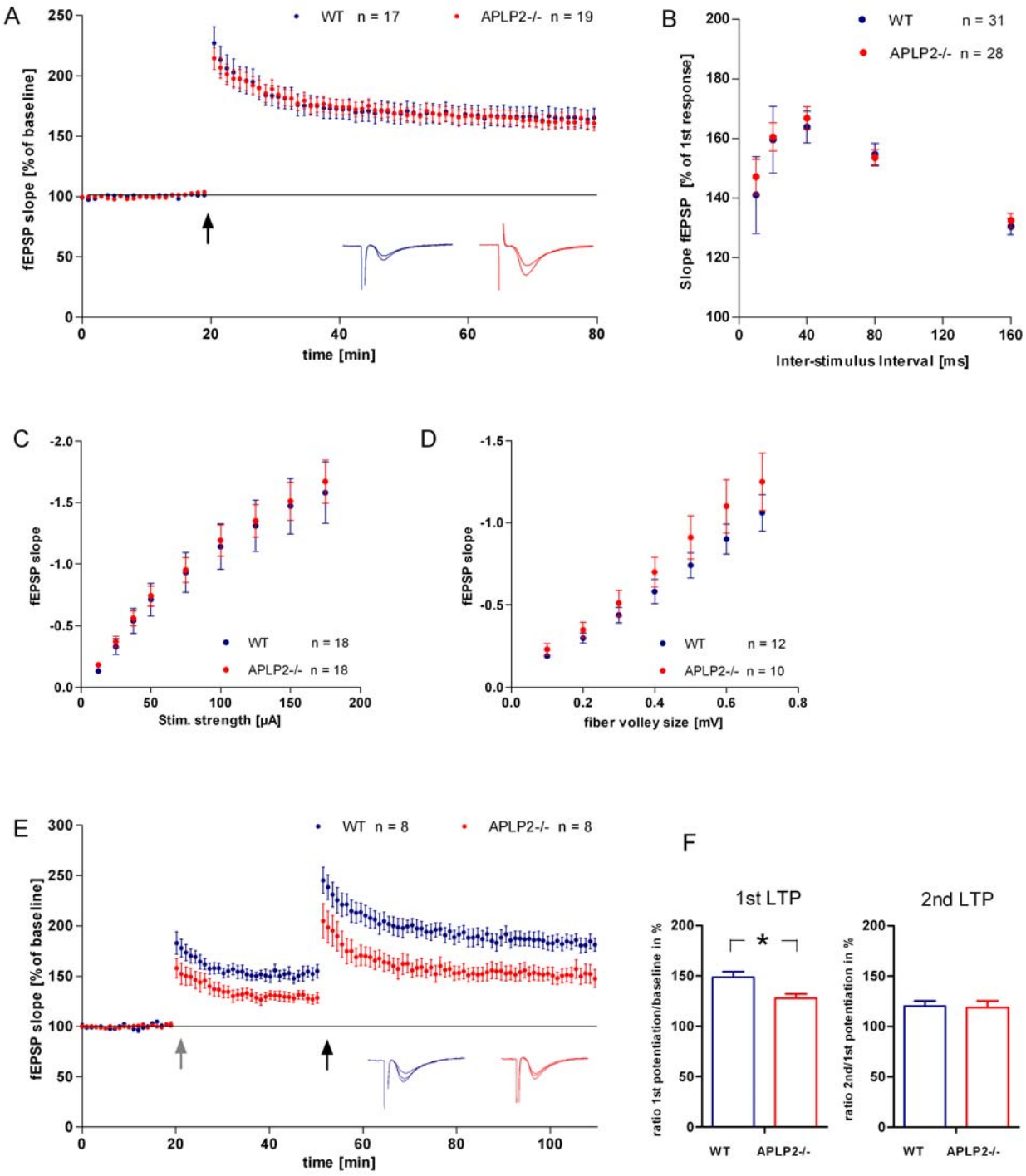


Figure 3.6: Unlike APP, deletion of the homolog APLP2 did not result in impairments in synaptic transmission or plasticity. **(A)** APLP2$^{-/-}$ mice of the same age (9-12 months) had unaffected abilities for LTP-induction (WT 164.9 ± 7.83; APLP2$^{-/-}$ 161.9 ± 5.97) by TBS (arrow). Representative fEPSPs are shown for both experiments before and, overlayed, 60 min after TBS. Basal synaptic transmission relative to stimulus strength **(C)** and to fiber volley size **(D)** and presynaptially mediated paired-pulse facilitation **(B)** also were both unaffected. Error bars: standard error of the mean.

(E) A special form of synaptic plasticity, primed LTP, showed differences between aged APLP2$^{-/-}$ and WT littermates. **(F)** Left graph: 30 minutes after the first weak theta burst stimulation (1x, grey arrow), LTP was significantly reduced in APLP2$^{-/-}$ (WT 153.6 ± 18.23; APLP2$^{-/-}$ 129.2 ± 12.67; $p = 0.008$). Right graph: 60 minutes after the second stimulus of regular strength (3 theta bursts, black arrow) induction rates in APLP2$^{-/-}$ were also significantly lower ($p < 0.05$), but the relative increase of fEPSP size from before to after the second stimulus was identical for both genotypes ($p = 0.855$). Error bars: standard error of the mean.

I also investigated a special form of synaptic plasticity, so called primed LTP (Fig. 3.6 E, F). Here, a weak plasticity-inducing stimulus "primes" active synapses for later regular stimulation. Concerning the mechanisms it has to be discerned between the first and the second stimulus. As the first stimulus is relatively weak, this stimulation protocol is more sensitive to detection of subtle deficits. The cellular responses to the second stimulus are believed to comprise additional factors, because the priming stimulus induced changes at the active pre- and postsynapses. For priming I used one theta burst train (white arrow) and for later stimulation three 100Hz theta burst trains (black arrow), the same as used for "regular" LTP induction. Slices of APLP2$^{-/-}$ mice showed significantly lower potentiation rates from 10 minutes after the weak stimulus on, that became even more pronounced after the second potentiation stimulus. The comparison of the response to the first stimulus to that in regular LTP experiments confirms that the regularly used protocol with three trains of theta burst stimulation is optimal for LTP induction, as the weak stimulation already causes almost the same rate of potentiation in wildtype animals.
However, the amounts of potentiation in response to the second stimulus do not differ in size, if the EPSP size before the potentiation is taken as reference. As shown in figure 3.6 F, potentiation rates in response to the strong stimulus did not differ between WT and APLP2$^{-/-}$ animals, confirming the results from the regular LTP experiments in these animals and implying that synapses were not primed differently and no hidden deficits in pre- or postsynaptic mechanisms were present. The difference between KO and WT animals selectively for weak stimulation, however, hints to a slightly higher induction threshold for LTP in APLP2$^{-/-}$ mice. This shows that APP and APLP2 do not have identical functions or at least identical relevance for the CNS synapse. It does however not exclude that APLP2 might serve other functions in the nervous system or that combined deletions might have an effect.

3.2.2 Mice with combined mutations for APLP2$^{-/-}$ and APPsα or APPΔCT15

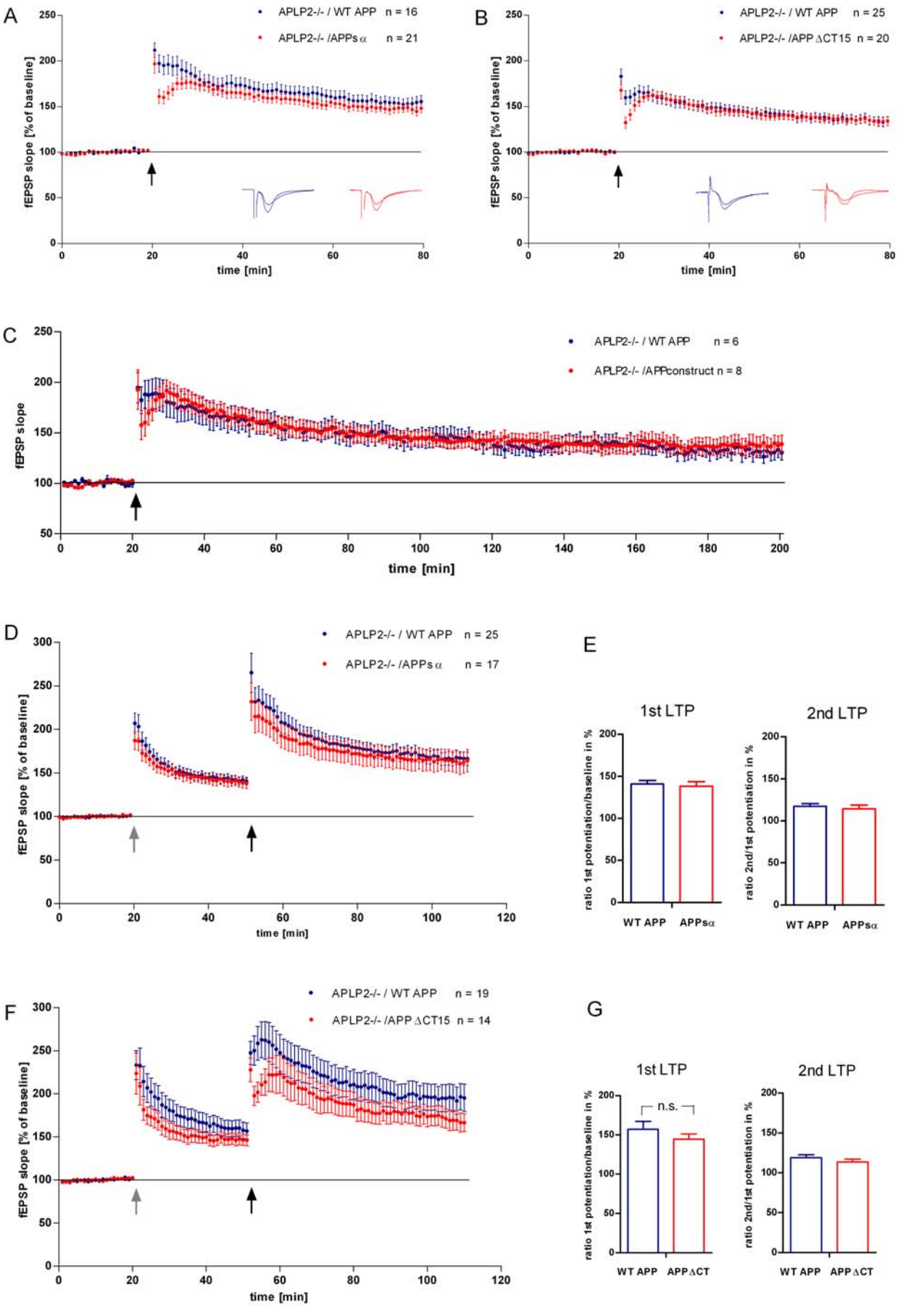
Unlike single deletions, a combined loss of APP and APLP2 is lethal (Heber et al., 2000), indicating at least partial functional redundancy of both molecules. Mice carrying double deletions for APP and APLP2 die shortly after birth (Heber et al., 2000; Bibel et al., 2007). To exclude confounding effects of APLP2 when further investigating the role of APP, in the lab of Prof. Müller APLP2$^{-/-}$ mice were crossed with mice expressing the truncated APP versions described in 3.1.2 instead of APP, resulting in litters containing homozygous APLP2$^{-/-}$/APPsα or APLP2$^{-/-}$/APPΔCT15 expressing animals respectively, APP$^{+/+}$/APLP2$^{-/-}$ individuals, which were used as controls and mice heterozygous for truncated APP constructs.
Both of these strains turned out to be viable, however lethality within the first month after birth is significantly higher in both strains for mice that are homozygous for the truncated APP forms. Animals are born at normal Mendelian frequency, yet during the first 30 days after birth

50% of truncated APP$^{-/-}$/APLP2$^{-/-}$ KI animals die and the surviving animals show reduced body weight (quantification S.Weyer, Heidelberg). Additionally, most KI mice show behavioral abnormalities, especially hyperactivity and circling and have reduced body weight. All deficits affect APLP2$^{-/-}$/APPΔCT15 expressing mice slightly less than APLP2$^{-/-}$/APPsα knock-in mice which remained noticeably weak throughout their life. For electrophysiological experiments on adult animals of both strains, only animals older than 12 weeks and displaying a behavioral phenotype were used.
As the behavioral abnormalities in both strains are present from the beginning of their life on, I investigated whether they are paralleled by alterations in synaptic and network activity that would be reflected in altered activity-dependent plasticity. I investigated long-term potentiation in animals of both strains of an age when they had reached a stable level of survival in order to avoid confounding effects because of a generally bad health state of the animals.

Animals of both strains showed completely unimpaired rates of LTP 60 minutes after induction (Fig. 3.7 A,B, next page). The outcome of behavioral learning experiments is still pending. There are striking differences during the first five minutes after TBS that will be dealt with in chapter 3.3. Transcription-dependent late-LTP was also unaltered (Fig 3.7 C). I again pooled experiments from both strains, as there was no visible difference in induction rates.
I also tested primed LTP to investigate whether the induction threshold for LTP in these animals was lowered. The induction following the weak theta burst stimulation did not yield significant differences for either strain as assessed by student´s t-tests for each time point. Neither did the ratio of potentiation by the second stimulus (Fig. 3.7 D-G). Two-way ANOVA, however, yielded a significant correlation between genotype and induction rate at minute 26 to 50 and 62 to 110 in APLP2$^{-/-}$/APPΔCT15 expressing mice.

Figure 3.7 (next page) Combined mutants, carrying truncated versions of APP and lacking APLP2 displayed unaltered LTP induction rates 60 minutes after TBS (arrow) at the age of 4 months compared to their littermates with full-length APP and without APLP2 ("WT APP"). **(A)** APLP2$^{-/-}$/APPsα strain (WT APP 153.9 ± 6.28; APPsα 147.5 ± 4.72) **(B)** APLP2$^{-/-}$/APPΔCT15 strain (WT APP 133.9 ± 4.76; APPΔCT15 133.7± 4.66). **(C)** The late, transcription-dependent component of LTP was also unaltered in strains with truncated forms of APP plus deletion of APLP2 (WT APP 164.9 ± 7.83; APPconstruct 161.9 ± 5.97). Here, data from both strains were pooled. Primed LTP in non-aged mice carrying truncated versions of APP plus deletion of APLP2 was mostly intact. **(D), (E)** APLP2$^{-/-}$/APPsα expressing mice had completely unaltered induction rates after both stimuli (grey arrow single TBS, black arrow 3x TBS). **(F), (G)** APLP2$^{-/-}$/APPΔCT15 expressing mice had no significant deficits in LTP induction, as determined by student´s t-test, but two-factor ANOVA yielded a significant correlation only between genotype and induction rate at min 26-50 and min 62-110. There was no significant further potentiation difference before and after the second stimulus.
Error bars: standard error of the mean.

Figure 3.7


A
APLP2-/- / WT APP n = 16
APLP2-/- /APPsα n = 21
fEPSP slope [% of baseline]
time [min]
B
APLP2-/- / WT APP n = 25
APLP2-/- /APP ΔCT15 n = 20
fEPSP slope [% of baseline]
time [min]
C
APLP2-/- / WT APP n = 6
APLP2-/- /APPconstruct n = 8
fEPSP slope
time [min]
D
APLP2-/- / WT APP n = 25
APLP2-/- /APPsα n = 17
fEPSP slope [% of baseline]
time [min]
E
1st LTP
2nd LTP
ratio 1st potentiation/baseline in %
ratio 2nd/1st potentiation in %
WT APP
APPsα
F
APLP2-/- / WT APP n = 19
APLP2-/- /APP ΔCT15 n = 14
fEPSP slope [% of baseline]
time [min]
G
1st LTP
2nd LTP
n.s.
ratio 1st potentiation/baseline in %
ratio 2nd/1st potentiation in %
WT APP
APP ΔCT

Given the lack of relevance of APLP2 for age-dependent effects and the fact that the APPsα fragment, that was found to be responsible for LTP-deficits in old age, was still present in both KI strains, it was unlikely that in aged animals of those strains LTP deficits should occur. I confirmed this exemplarily in aged APLP2$^{-/-}$/APPΔCT15 knock-in mice, inducing LTP by 100Hz tetanus (Fig. 3.8 A) and by 50 Hz theta burst stimulation (Fig. 3.8 B). The latter is more gentle than the 100Hz theta burst stimulation, causing lower depolarisation and Ca^{2+} influx. Theta burst stimulation and tetanus also differ in their partial dependence on different induction mechanisms. Neither protocol resulted in differences in LTP induction.

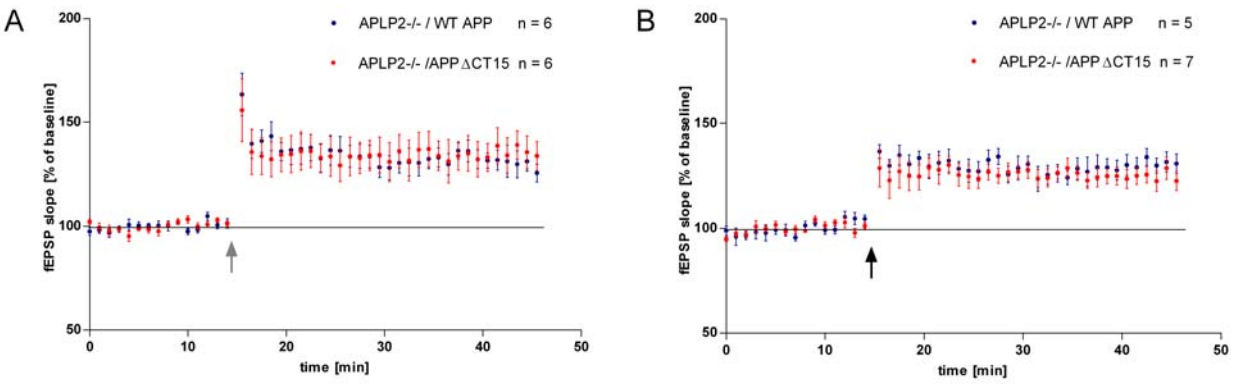


Figure 3.8 Aged APLP2$^{-/-}$/APPΔCT15 mice still have intact LTP. This was confirmed by 100 Hz Tetanus (3x30 pulses, grey arrow) (WT APP 129.8 ± 5.61; APPΔCT15 135.5 ± 7.56) **(A)** and theta burst stimulation using 3 trains consisting of bursts of 50 Hz frequency (black arrow) (WT APP 138.1 ± 7.98; APPΔCT15 137.4 ± 13.21 **(B).**

These results allow two immediate conclusions. Firstly, they show, that the behavioral and synaptic (see below) phenotype of both strains did not lead to age-dependent deficits in long-term potentiation in the hippocampus. Secondly, in combination with the findings on mice with intact APLP2, they demonstrate that there was no additional role of APLP2 in LTP-relevant functions, preventing LTP deficits in young animals.

3.3 The role of the APP and APLP2 intracellular domain for synaptic function

The experiments described above revealed a function of the secreted ectodomain of APP in preserving the ability for activity-dependent synaptic plasticity in age. The following experiments shed light on the significance of the intracellular domains of APP (AICD) and APLP2 (ALICD).

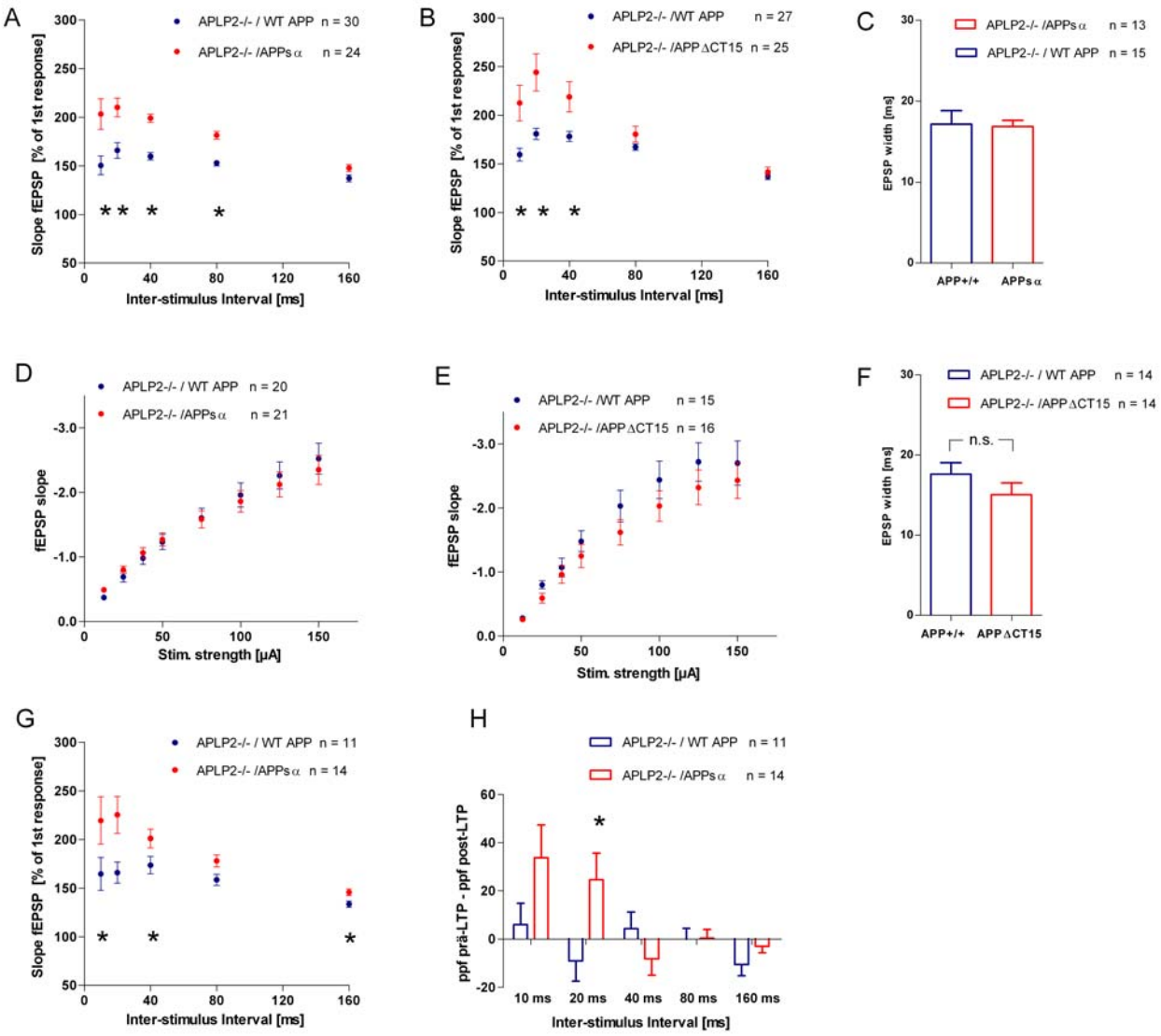


Figure 3.9 Paired pulse facilitation (PPF) was significantly increased in both KI strains while basal synaptic transmission was unaltered. Ratios of fEPSP slopes in CA1 region in response to two closely spaced stimuli are shown for different inter-stimulus intervals (ISI). APLP2$^{-/-}$/APPsα expressing mice **(A)** and APLP2$^{-/-}$/APPΔCT15 mice **(B)** showed significantly ($p<0.05$ - $p<0.0005$, Student´s t-test) higher responses to the second pulse than APLP2$^{-/-}$/APP$^{+/+}$ littermates. Basal synaptic transmission, plotted as slope of the fEPSP against the strength of the stimulus to the Schaffer Collaterals, was unchanged (**(D)** APLP2$^{-/-}$/APPΔCT15 strain **(E)** APLP2$^{-/-}$/APPsα strain). The duration (width) of the second field EPSP at an ISI of 160 ms was similar for both genotypes in the experiments **(C, F)**. The amount of paired-pulse facilitation altered in APLP2$^{-/-}$/APPsα-KI after LTP induction **(G)** shows PPF results in APLP2$^{-/-}$ /APPsα mice 60 minutes after LTP-induction. In **(H)**, the development of PPF is shown as facilitation before LTP divided by facilitation after LTP. All data are presented as mean ± SEM.

APPΔCT15 expressing strains with and without APLP2$^{-/-}$ background lack the last 15 amino acids of APP containing the intracellular YENPTY interaction motif. The APPsα expressing strain additionally lacks the whole AICD plus the transmembrane domain. Whereas mice expressing truncations of APP, but intact APLP2 did not show any apparent phenotype, mice with APLP2$^{-/-}$ background displayed several alterations.

In both strains, the amount of paired pulse faciliation was significantly enhanced in animals lacking the C-terminal end of the AICD (Fig. 3.9 A,B). The input-output relationship between stimulus strength and EPSP sizes was not altered (Fig. 3.9 D,E). So, during paired pulse stimulation, the first response was of similar size, whereas the second answer was significantly higher in mice expressing APP truncations instead of WT APP. Pulse pairs were presented in 10 second intervals. So, after 10 seconds, resting states at the CA3-CA1 presynapse had been re-established again.

Paired-pulse facilitation characterizes the fEPSP increase in response to the second of two successive pulses within the range of milliseconds and as described above in 4.1.1 is usually applied to assess the functionality of the presynaptic terminal. The influence of increased neurotransmitter release is largest at inter-stimulus intervals of 10 ms, corresponding to 100Hz stimulation and decreases with lower stimulus frequencies. The increase in facilitation ratio was largest for inter-stimulus intervals of 10 and 20 ms or 100 and 50 Hz, respectively (table 3.1).

Table 3.1 Percentaged increase of PPF ratio in both transgenic strains was largest at stimulus frequencies of 100 and 50 Hz.

Inter-stimulus interval	Frequency	% increase in PPF ratio	
		APLP2$^{-/-}$/APPsα	APLP2$^{-/-}$/APPΔCT15
10	100Hz	35.02	33.02
20	50 Hz	26.84	34.90
40	25 Hz	24.55	22.78
80	12.5 Hz	18.69	7.92
160	6.25 Hz	7.66	3.41

EPSPs also experience shortening by the inhibitory postsynaptic potentials (IPSPs) of inhibitory interneurons, stimulated by the Schaffer Collaterals and forming synapses onto CA1 pyramidal neurons. The feedback suppression of inhibitory (GABAergic) transmitter release by metabotropic $GABA_B$ receptors is visible indirectly in widening of the second EPSP in the time-range of 100 – 2000 ms. The width of the second fEPSP at an inter-stimulus interval of 160 ms was unchanged, arguing against altered IPSPs (Fig. 3.9 C,F).

The difference in facilitation decreased after LTP induction. In a set of experiments on APLP2$^{-/-}$/APPsα expressing animals, I measured PPF not only before, but also after LTP induction (Fig. 3.9 A,G). To avoid reaching the maximal level of the synapse, I adjusted EPSP levels to 40% again after completion of the LTP experiment and subtracted the facilitation value after LTP induction from that before LTP induction. 60 minutes after theta burst stimulation,

facilitation rates in APLP2$^{-/-}$/APP$^{+/+}$ expressing animals had stayed similar, whereas facilitation rates in slices from APLP2$^{-/-}$/APPsα expressing animals decreased after LTP induction, albeit still larger than in WT APP littermates. At an inter-stimulus interval of 20 ms the difference was significant (p=0.05). Figure 3.9 H shows how much the first PPF was larger than the second. Please note that this difference cannot be inferred by direct comparison of the PPF graphs, as the data sample of PPF before LTP induction contains additional experiments.

A third indication of altered synaptic or network function is provided by the differences in fEPSPs between WT and KI animals during the first minutes after theta burst stimulation. Figure 3.10 shows the alteration of EPSPs five minutes before until 15 minutes after theta burst stimulation at larger scale.
Here, each data point represents a single sweep from each experiment, different from the earlier LTP analyses, where each data point represented values, averaged over six sweeps, for each experiment. More obvious than in the smaller scale figures, fEPSP levels were significantly lower in KI mice during the first five minutes after theta burst stimulation, but after this time-window long-term potentiation reaches the same level.

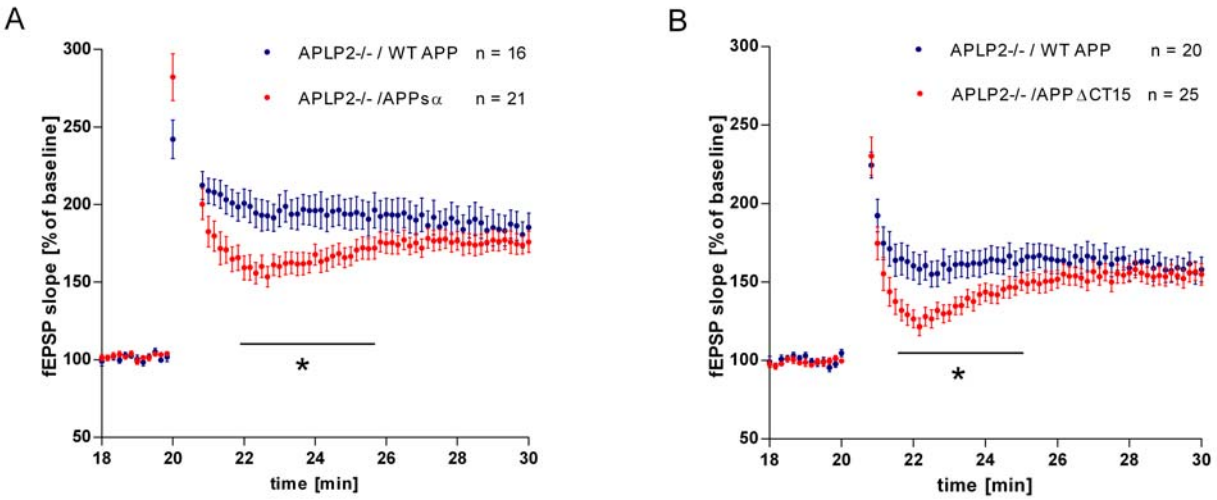


Figure 3.10 Different transient changes of fEPSPs immediately after theta burst stimulation. In both strains expressing truncated APP plus no APLP2, fEPSP responses decreased transiently after TBS before reaching identical potentiation levels as WT littermates. **(A)** APLP2$^{-/-}$/APPsα, significant differences from 21.5 to 25.1 minutes after TBS, **(B)** APLP2$^{-/-}$/APPΔCT15, significant differences from 21.3 to 24.3 minutes after TBS. Significances were determined with student´s t-test.

Preliminary data indicate that this phenotype becomes more pronounced when applying a theta burst stimulation protocol, using not 100Hz bursts, but 50 Hz bursts. This corresponds to 20 ms inter-stimulus intervals that yielded the largest differences in facilitation during pulse pairs between animals expressing wildtype APP and APP truncations. Figure 3.11 A shows an exemplary experiment in a 2-months-old APLP2$^{-/-}$/APPΔCT15 individual, in which

fEPSP slopes decreased to 50% of baseline level before rising above 100%. Data points represent the responses to single pulses.

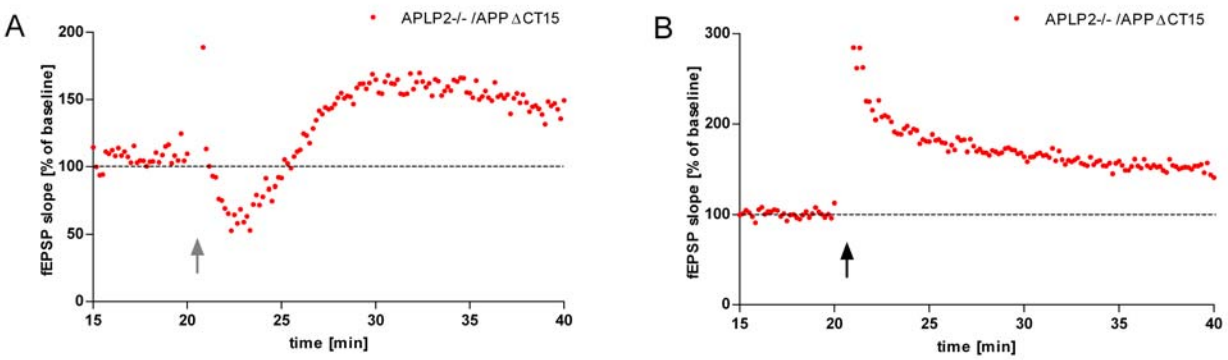


Fig.3.11 Responses to different stimulus protocols in APLP2$^{-/-}$/APPΔCT15 mice. After application of a 50 Hz TBS, fEPSPs in were transiently depressed. In an exemplarily shown experiment, fEPSPs reached a minimum of 50 % of the averaged baseline 5 minutes after TBS **(A)**. After application of a 100 Hz Tetanus, post-tetanic potentiation was fully present **(B)**. Data points represent responses to single stimuli presented with a frequency of 0.1 Hz.

In aged APLP2$^{-/-}$/APPΔCT15-KI animals, however, this phenotype was not reproducible (Fig. 3.8 B). After induction of a 50 Hz TBS there was a lack of short term potentiation in both genotypes, but in none of the single experiments on KI animals there was an additional collapse of the EPSP as observed in young animals.

So, these experiments show that the simultaneous lack of the AICD of both APP and APLP2 age-independently altered distinct aspects of transmission at the CA3-CA1 synapse. The requirement for combined lack of the AICD and ALICD stresses the notion of functional complementation of APP and APLP2 for this aspect of synaptic transmission.

3.4 Investigation of involvement of APP in negative synaptic plasticity

So far I could demonstrate a role of APP in activity-dependent positive synaptic plasticity, i.e. enhancement of synaptic responses that critically depended on APPsα, the product of the α-secretase activity. The following experiments were performed to specify a role of APP in the opposite phenomenon, in long-term depression (LTD).
I investigated two kinds of *de novo* LTD that differ by intracellular mechanism and induction protocol. LTD induced by 15 minutes of low-frequency (1Hz) stimulation is dependent on activation of the NMDA receptor and only inducible during a time window roughly between

p14 and p21 (Kemp et al., 2000; Kemp and Bashir, 2001) (single-pulse LTD). Stimulation for 15 minutes with double pulses of 1 Hz frequency and an inter-pulse interval of 50 ms induces a form of LTD that is NMDA receptor independent presumably inducible throughout life (Bortolotto et al., 1999; Kemp et al., 2000) (paired-pulse LTD).

Induction of LTD by application of 900 single pulses at 1 Hz resulted in identical rates of LTD in APP KO and WT animals in both age-groups p14 to p17 (Fig. 3.12 A, next page) and p20 to p22 (Fig. 3.12 B). The rate of LTD decreased continuously with each postnatal day (Fig. 3.12 C), however similarly in APP expressing and $APP^{-/-}$ animals. Data points in figure 3.12 represent the mean values of induction rates between 55 and 60 minutes after LTD induction for all experiments of a given postnatal day from the analysis in figure 3.12 A and B. In order to exclude biasses in the genotype groups, I exactly age-matched the experiments, including similar numbers of experiments per day for both genotypes. Linear regression analysis yielded no significant differenes in slope ($P<1.0$) or elevation and intercept ($P<0.7$) for both regression lines and 95% confidence intervals yielded largely overlapping areas for the course of the regression lines.

Non-NMDA receptor dependent LTD was investigated by applying 900 pulse pairs at 1 Hz with 50 ms inter-pulse interval in p20 animals. This form of LTD was still well inducible and there was no deterioration of induction rates (Fig. 3.12 E). Application of the same protocol in the p14-p17 age-group induced an NMDA receptor dependent form of LTD, as confirmed by application of APV (Fig. 3.12 D). Figure 3.12 F depicts the distribution of mean induction rates on respective postnatal days. No regression curves are into the data, as the four data samples are too small to yield meaningful curves.

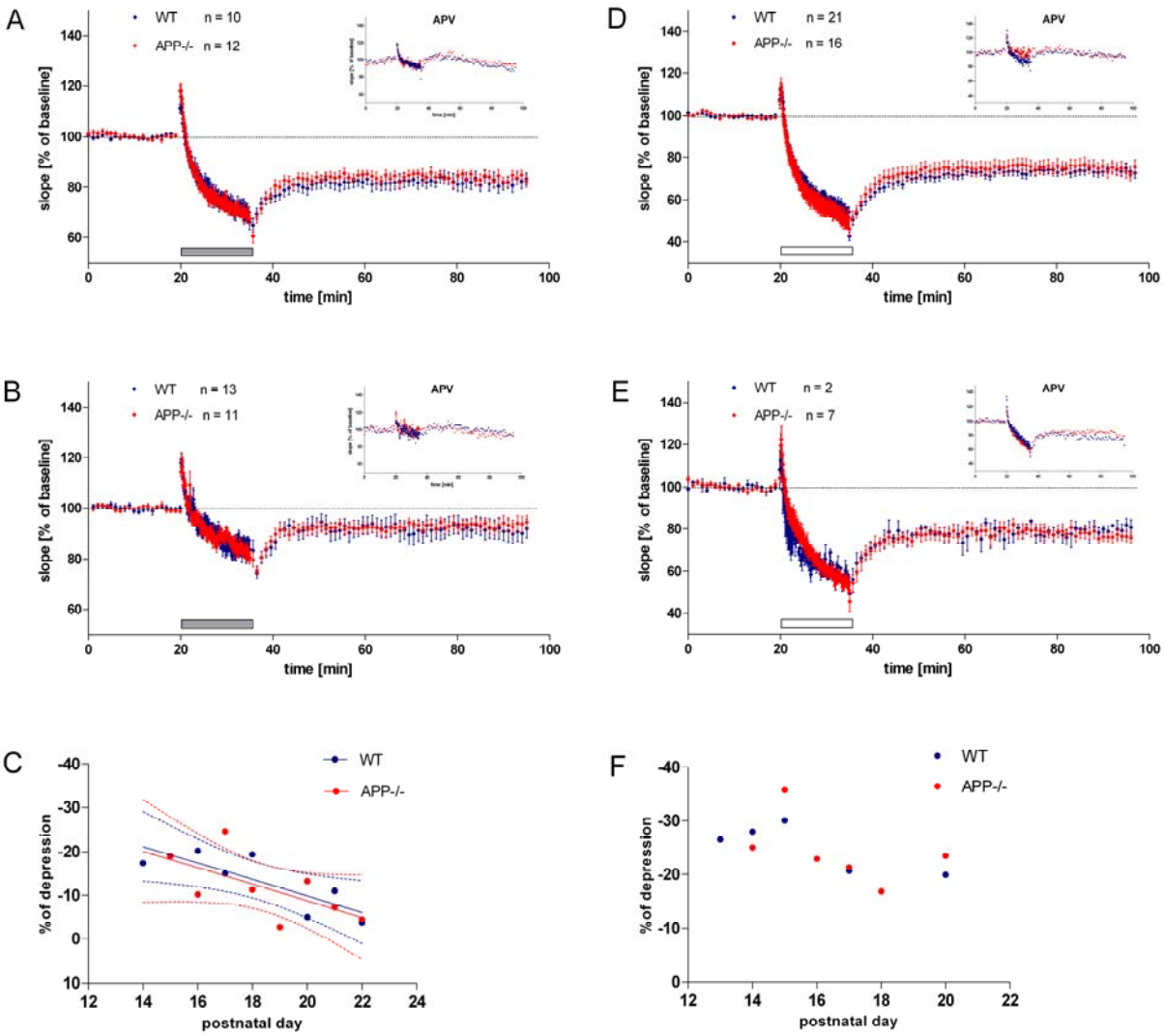


Figure 3.12 LTD induction by different protocols did not change with genotype, but over time.
Left column: NMDAR-dependent LTD was unimpaired in juvenile animals lacking APP. Neither in the age-group between p14 and p17 (WT 86.5 ± 11.73; $APP^{-/-}$ 83.5 ± 7.36) **(A)** nor in the group of p20 to p22 (WT 90.8 ± 13.77; $APP^{-/-}$ 93.8 ± 8.55) **(B)** there were significant differences. LTD induced by 900 single pulses at 1 Hz (grey bars) was NMDAR-dependent throughout, as confirmed by addition of APV (inserts). The induction rates for NMDAR-dependent LTD decreased with each postnatal day, but did not show a different time course in APP KO and WT animals as determined by linear regression and 95% confidence intervals **(C)**.
Right column: LTD induced by a paired-stimulus protocol (white bars) was unimpaired in juvenile animals lacking APP, but induction mechanisms activated by the protocol changed. Neither in the age-group between p14 and p17 (WT 74.2 ± 10.34; $APP^{-/-}$ 74.0 ± 12.47) **(D)**, nor in the group of p20 (WT 80.5 ± 4.17; $APP^{-/-}$ 76.5 ± 6.56) **(E)** there were significant differences in LTD induction. However, in p14 - p17 animals, stimulation by 900 pulse pairs at I Hz induced a purely NMDAR-dependent form of LTD as determined by blocking of the NMDAR during LTD induction with APV (insert). At p20, a putatively mGluR-dependent form of LTD was induced. **(F)** gives an overview over the distribution of mean induction rates over development.

In $APP^{-/-}$ mice, all cleavage products of APP are absent. Human Aβ has been shown to promote LTD, but APPsα also has been proposed to impair LTD by raising the induction threshold (Ishida et al., 1997), with the consequence that lack of both might induce effects that cancel each other out. In order to prevent potential masking effects, I repeated analysis of

NMDAR-dependent LTD on mice from the APLP2$^{-/-}$/APPsα strain and the APLP2$^{-/-}$/APPΔCT15 strain. Both strains are also lacking the AICD, composing the other active fragment, resulting from β- and γ-secretase cleavage. APLP2$^{-/-}$/ APPΔCT15 expressing mice were also tested as they had shown a slight tendency towards altered LTP function.

In both strains, no alterations in NMDA receptor dependent long-term depression were detectable in any of the age-groups (Fig. 3.13), showing that neither lack of the AICD alone played a role in NMDA receptor dependent LTD in these mice, nor had the lack of APPsα in APP$^{-/-}$ mice masked an alteration of NMDA receptor dependent LTD.

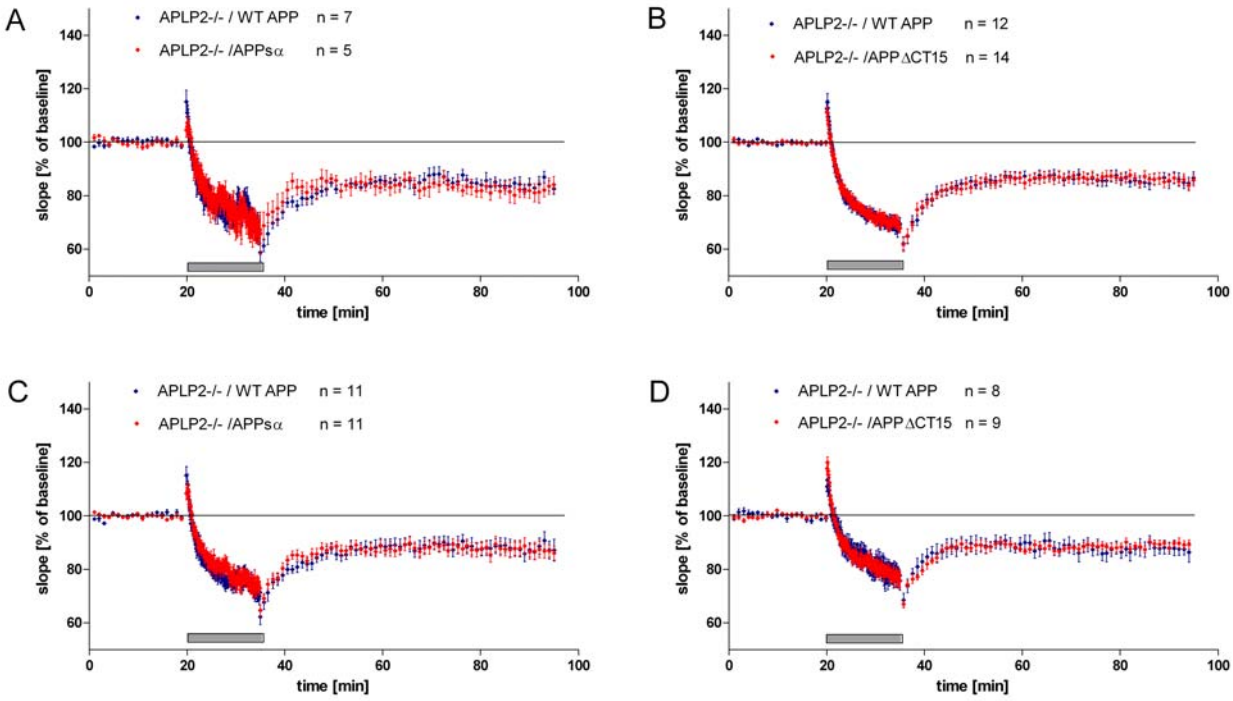


Figure 3.13. NMDAR-dependent LTD was unimpaired in both KI strains. LTD induced by 900 single pulses at 1 Hz (grey bars) yielded identical LTD induction rates in APLP2$^{-/-}$/APPsα-KI **(A,C)** and APLP2$^{-/-}$/APPΔCT15-KI **(B,D)**.

4 Discussion

Comprehension of the pathophysiological processes leading to a disease is not possible without an understanding of the physiological roles of the involved proteins. Therefore, to understand the mechanisms leading to Alzheimer`s Disease it is necessary to know the functions of APP, its homologues and active fragments in the intact organism.
I focussed on the three major domains and cleavage products of APP: the ectodomain APPsα, the Aβ fragment and the intracellular domain AICD. All experiments were performed on the model of the hippocampal CA3-CA1 synapse. I analyzed field recordings in respect to synaptic function, short-term and long-term plasticity and complemented them with pharmacological manipulation. To overcome the problem of potential functional compensation of the lack of APP by APLP2, I performed corresponding experiments in mouse strains expressing combinations of knock-out of APLP2 plus mutated versions of APP.
The main findings of my thesis were the following.

A) Aged mice lacking APP had impaired long-term potentiation. This impairment was rescued in aged mice expressing only the APPsα domain of APP. The difference in LTP induction was also abolished by blocking $GABA_A$ receptor mediated inhibitory synaptic transmission. The role of the respective domain of the APP homologous protein APLP2 for intact LTP in aged animals was considerably lower.

B) Lack of the APP intracellular domain AICD did not lead to defects in synaptic function or plasticity in aged mice. In cases of simultaneous lack of APLP2, however, short-term plasticity was affected independently from age. Animals showed enhanced paired-pulse facilitation and a transient decrease of synaptic responses after LTP induction. LTP itself was not affected.

C) Lack of APP and especially of the Aβ fragment was not found to alter synaptic plasticity in the form of long-term depression. Compensatory effects of APPsα were excluded in experiments on mice expressing this part of APP without Aβ.

In the following, each of the findings will be discussed in the context of the current literature and under consideration of methodological issues.

4.1 The soluble ectodomain of APP as mediator of synaptic integrity

4.1.1 LTP deficit in aged APP knock-out mice

To determine whether APP is linked to hippocampal synaptic plasticity, long-term potentiation (LTP) was compared in hippocampal slices of aged (9-12 months) wildtype and $APP^{-/-}$ mice and revealed a robust deficit in induction as well as maintenance of LTP in mice lacking APP. After 60 minutes, levels of potentiation in aged $APP^{-/-}$ mice reached only half of the values of wildtype littermates (Fig. 3.1). Young animals showed no deficits.

A very moderate deficit in LTP had been described in aged individuals of a line of $APP^{-/-}$ mice that had existed several years ago (Seabrook et al., 1999; Dawson et al., 1999), but these animals had been suffering from severe gliosis (Zheng et al., 1995; Dawson et al., 1999). Accumulation of astrocytic glial cells indicates the existence of neunal damage (Trendelenburg and Dirnagl, 2005). Also, astrocytes were recently shown to actively participate in synaptic transmission and plasticity by taking up and releasing glutamate (Haber et al., 2006; Jourdain et al., 2007). So, in that strain of mice, it had not been possible to exclude that the pathological astrocytical phenotype had contributed to the LTP deficit. The mice used here, however, showed no visible signs of gliosis and had unaltered numbers of pyramidal neurons in areas CA3 and CA1. On the functional level, basal synaptic transmission and also short-term plasticity as assessed by comparison of fEPSP sizes and ratios of paired pulse facilitation were intact, indicating no loss of excitatory synapses or alteration of presynaptic function. A recent paper had found unchanged release probability, but a larger pool of readily releasable vesicles in hippocampal autaptic cultures of $APP^{-/-}$ mice (Priller et al., 2006). This strengthens our finding, because the latter was likely to be influenced by the particular experimental system. In preparations of the mature neuromuscular junction of $APP^{-/-}$ mice, synaptic transmission was also found to be normal (Yang et al., 2007).

Aged $APP^{-/-}$ mice also showed impaired performance in memory-dependent behavioral tasks, paralleling the LTP deficit (Ring et al., 2007). This demonstrates firstly, that specifically processes associated with activity-dependent synaptic plasticity were impaired by the life-long lack of APP, yet not the general functionality of the excitatory CA3-CA1 synapse. Secondly, it also showed that, as mice with impaired LTP and memory function did not produce Aβ, APP itself must have an important, possibly protective role for learning and memory. This justifies the hypothesis that if other parts of APP have a (positive) influence on memory function, the memory deficits observed in AD might result at least partly from lack of these.

4.1.2 Rescue of the LTP deficit by the APPsα domain

In order to specify the responsible part of APP, I examined gene-targeted mice expressing truncated versions of APP instead of wildtype APP. These mice either expressed mutated APP lacking a functional APP intracellular domain (AICD) or a variant corresponding to the soluble ectodomain of APP which in the wild-type organism is generated by α-secretase cleavage (APPsα). In both strains, even older (12-15 months) mice showed unaltered rates of LTP compared to wild-type littermates (Fig. 3.2). This allows the conclusion that the LTP defect observed in aged $APP^{-/-}$ mice was caused by a loss of physiological functions of the soluble APPsα fragment. The intact LTP was paralleled by a rescue of memory function, assessed by Morris water maze acquisition learning (Ring et al., 2007). This finding was surprising, as putative functions of holo-APP, implicated by its resemblance to cell surface receptors (reviewed in Reinhard (2005)) could now be excluded as mediators of intact LTP.

Earlier experiments employing anti-APP antibodies or antisense RNA had hinted to an involvement of APP in learning and memory (Turner et al., 2003), but the interpretation had been hampered by the fact that the antibodies used could not distinguish between secreted and full length APP and may also have cross-reacted with APP homologous proteins. Some studies were able to directly ascribe a morphoregulatory function to the shedded APP ectodomain, documenting actions on neurite outgrowth (Milward et al., 1992; Young-Pearse et al., 2008) and synaptogenesis (Moya et al., 1994; Morimoto et al., 1998) in neural cell culture by addition of APPsα.

A more specific approach was undertaken in experiments stimulating α-secretase activity in a mouse model for AD. Here, decrease of AD like pathology was described after overexpression of the α-secretase ADAM10 (Postina et al., 2004; Postina, 2008). In wildtype mice, enhanced cortical synaptogenesis was reported after ADAM10 overexpression (Bell et al., 2008). Conversely, levels of APPsα, ADAM10 and neuronal activity have been found to be reduced in cases of sporadic AD (Lannfelt et al., 1995; Almkvist et al., 1997; Sennvik et al., 2000; Colciaghi et al., 2002; Tyler et al., 2002). These decreased APPsα levels could be associated with deficits in CNS function in AD and aging (Lannfelt et al., 1995; Nistor et al., 2007). Likewise, in aged rats, APPsα levels in CSF have been found to correlate with spatial memory deficits (Anderson et al., 1999). High levels on the other hand correlate with successful aging. This is consistent with the observation that during aging and the development of AD, profound changes in APP processing reducing α-secretase cleavage occur. Accordingly, enhancement of α-secretase cleavage is being proposed as therapeutical target in AD (Fahrenholz and Postina, 2006; Bandyopadhyay et al., 2007).

However, a fundamental problem of all these studies is that by intervening with the cleavage equilibrium, the amounts of all cleavage products are altered. Although these results confirm that due to the competing nature of the proteases, upregulation of α-secrease cleavage

simultaneously reduces amyloidogenic cleavage and thereby plaque load, they do not allow conclusions about actions of APPsα itself, because these cannot be distinguished from effects of the reduced amyloid load. This holds especially true as in mouse models of AD, animals express the more amyloidogenic human APP. Ishida and colleagues avoided intervention with the cleavage balance by pre-incubating slices with APPsα and could report facilitated LTP (Ishida et al., 1997). However, they applied amounts of APPsα that exceeded those found normally in an organism. The same was the case in another study reporting memory-enhancing effects of APPsα upon infusion into the ventricles (Meziane et al., 1998).
In the mouse model I used, I circumvented this problem. Not only did I work in a system of murine APP and unaltered expression levels, but the main advantage of the experimental approach lay in the fact that the usually parallel down-regulation of APPsα and upregulation of Aβ could be dissociated. So I could show in an *in vivo* approach that aged $APP^{-/-}$ mice, lacking all possible cleavage products had an LTP deficit compared to wildtype mice, which was rescueable by APPsα alone, showing that the deficit in LTP observed in aged $APP^{-/-}$ mice is due to a loss of physiological APPsα function. This allows to assign an age-relevant role also to APPsα.
Still unknown is how APPsα mediates this age-dependent function, because apart from integrin binding (Young-Pearse et al., 2008) no receptor for APPsα has been found. I also could not specify the source of APPsα. In wild-type animals, APP cleavage in neurons follows predominantly the amyloidogenic pathway and the main source of APPsα are glial cells (Thinakaran and Koo, 2008). This may be different in APPsα-expressing mice, in which also neurons can only shed APPsα.

4.1.3 Involvement of GABAergic signaling

Excitatory synaptic transmission was not impaired in aged $APP^{-/-}$ mice. However, this did not rule out alterations of inhibitory synaptic transmission as underlying cause for the LTP deficit. Inhibitory neurons in the hippocampus shape neuronal signaling and influence activity-dependent plasticity of synaptic contacts by secreting GABA (γ-aminobutyric acid) that acts via ionotropic $GABA_A$ and metabotropic $GABA_B$ receptors. Decreased levels of GABA as well as loss of GABAergic neurons have been found in AD patients (Lanctot et al., 2004; Lanctot et al., 2007), in rodent models of AD (Ramos et al., 2006) and aging (Vela et al., 2003).
Under blockade of GABA mediated contributions to the induction of LTP by the receptor antagonist pictrotoxine, I found the LTP deficit between wildtype and $APP^{-/-}$ mice to be abolished (Fig. 3.5).

LTP was induced by theta burst stimulation, a protocol in which the critical depolarization permitting NMDA receptor activation is enabled, because GABA depresses its own regular release specifically during high-frequency synaptic transmission, see also introduction, page 7f. Schaffer Collateral axons innervate GABAergic interneurons in area CA1 simultaneously with pyramidal neurons. At low rates of synaptic transmission, $GABA_A$ receptor mediated hyperpolarisation intensifies the Mg^{2+} block of NMDA receptors (Collingridge et al., 1988). During intermittent high-frequency stimulation subsequent IPSPs are inhibited, because part of the released GABA feeds back to release-inhibiting metabotropic $GABA_B$ autoreceptors (Davies et al., 1991), a mechanism that is effective only during intermittent stimulation (Davies and Collingridge, 1993; Davies and Collingridge, 1996) So, theta burst stimulation activates feed-forward GABAergic interneurons, leading to mostly $GABA_A$-mediated hyperpolarisation of the postsynaptic excitatory neuron, but simultaneously to more important $GABA_B$-mediated depression of subsequent $GABA_A$-mediated IPSPs. This enables sufficient depolarization of the postsynaptic membrane to allow voltage-dependent NMDA receptor-mediated currents (Davies et al., 1991).

Two questions emerge concerning the nature of the alteration: firstly, which part of the mechanism was affected in knock-out mice and secondly, whether it had increased or decreased inhibition.

The fact that lack of $GABA_A$-mediated fast inhibition during LTP induction abolished the difference in LTP between wildtype and $APP^{-/-}$ animals shows that in aged $APP^{-/-}$ mice GABAergic neurotransmission via the $GABA_A$ receptor was altered. This can be caused by specifically affected function or prevalence of the receptor, altered feedback inhibition at GABAergic terminals or generally lower number or activity of GABAergic neurons and synapses. Specification of the nature of the alteration requires immunohistological staining of GABAergic terminals and analysis of miniIPSCs.

Affected $GABA_A$ receptor mediated synaptic transmission has been reported to cause both increased and decreased levels of theta burst induced LTP. Papadopoulos and colleagues reported lowered induction thresholds in mice with reduced $GABA_A$ receptor function (Papadopoulos et al., 2007). On the other hand, Seabrook, Fitzjohn and co-workers found decreased LTP rates in their aged APP knock-out mouse strain (Seabrook et al., 1999; Fitzjohn et al., 2000). Both phenotypes were abolished by addition of a $GABA_A$ receptor antagonist. The seemingly opposing findings can be reconciled in the light of the role both GABA receptors play in long-term potentiation. Decreased rates of theta burst-induced LTP are only possible, if also $GABA_B$ receptor function is affected. Higher LTP can still be due to an overriding effect of the lack of $GABA_A$ mediated initial inhibition.

There are indications for reduced overall GABAergic signaling in $APP^{-/-}$ mice. In the other $APP^{-/-}$ mouse strain, GABAergic signaling was altered in several respects: $GABA_A$

receptor-mediated inhibition was chronically reduced and a reduction in $GABA_B$ autoreceptor-mediated paired-pulse depression of IPSPs was observed (Seabrook et al., 1997; Fitzjohn et al., 2000).
A selective degeneration of subpopulations of GABAergic neurons was also found in mice with the APP Swedish mutation generating more β-secretase cleavage products (Ramos et al., 2006). This goes in line with reports of lower plasma levels of GABA in AD patients (Lanctot et al., 2007) and beneficial actions of GABA agonists on AD patients in some cases (Lanctot et al., 2004). However, the consequences of reduced GABA release are not clear as opposite alterations in GABAergic signaling have also been observed. Again in Swedish mutant mice, inhibition was found to be enhanced, due to upregulation of the α1 $GABA_A$ receptor subunit (Yoshiike et al., 2008). The authors could rescue LTP deficits by 10 day treatment of the mice with a $GABA_A$ receptor antagonist and concluded that memory impairment in aged or mutant mice stems from upregulation of GABAergic inhibition, mediated by amyloidogenic cleavage products and mirroring premature aging processes.
For aging generally, mostly upregulation of net GABAergic signaling is described. In aged healthy mice, similar alterations of the pharmacological properties of the hippocampal $GABA_A$ receptor and the modified expression of several subunits, increasing GABAergic inhibition have been reported (Vela et al., 2003). Whereas specific hippocampal interneuronal subpopulations were differentially reduced, this was again compensated by upregulation of the expression of the α1 $GABA_A$ receptor subunit.
So, in AD mouse models as well as in aging, loss of GABAergic neurons is paralleled by altered activity of the remaining neurons, resulting in complex alterations of GABAergic network contributions. As the deficit is equally rescued in APPsα expressing mice and in both cases APPsα levels are reduced, it is likely that the same effects are present in aged $APP^{-/-}$ mice. The mechanisms involved still need to be elucidated.

4.1.4 Overlapping functions of the ectodomain of APLP2

The fact that the LTP deficit in $APP^{-/-}$ mice was fairly mild and only occurred in aged animals had led to the speculation whether stronger deficits were masked by intact function of the APP homologue APLP2. Both proteins are evolutionarily conserved, identical apart from the Aβ domain, share similar expression patterns and are processed alike (Walsh et al., 2007). Involvement of APLP2 in synaptic plasticity, alone or together with APP, is not described yet. So possibly APLP2 had compensated least partly for the lack of the other.

Examination of LTP in $APLP2^{-/-}$ mice of the same age as old $APP^{-/-}$ mice animals did not reveal an LTP deficit under regular LTP induction parameters (Fig. 3.6). This was paralleled by

intact memory function as assessed in behavioral testing (personal communication Prof. D. Wolfer, ETH Zürich) which shows that APLP2 does not have the same relevance for synaptic plasticity as APP and especially, that the lack of the ectodomain of APLP2 (APLP2sα) plays no or a minor role in respect to age-dependent alterations of synaptic plasticity and memory compared to APPsα. This was unexpected, as APLP2 is the only member of the mammalian APP family that can simultaneously compensate for the lack of APP or APLP1 (Anliker and Mueller, 2006).
Application of a very weak induction protocol (one train of theta bursts instead of three and starting from weaker baseline stimulation), however, did result in lower LTP in aged APLP2$^{-/-}$ mice compared to wildtype littermates. A weaker induction protocol is more sensitive to detection of small deficits, as regular protocols are designed to reliably induce LTP. This is mirrored in the fact that in wildtype mice, LTP induced by one theta burst train was only slightly lower than LTP induced by three trains. With weaker stimulation the induction becomes more difficult, because the postsynaptic depolarisation is not strong enough to depolarize the cell sufficiently to remove the Mg^{2+} block from the NMDA receptor (representing the requirement of cooperativity). This indicates a subtly altered induction threshold for LTP in aged APLP2$^{-/-}$ animals that was passed by the strong protocol, but only partly by the weak protocol, corresponding to a right shift of the stimulation/potentiation function. To determine whether the ability for LTP is generally lowered or only the induction threshold is shifted, experiments aiming for saturation of LTP would be necessary.

The reasons are not yet clarifyed. APLP2$^{-/-}$ mice recently have been found to have moderately reduced expression α1 subunit of the $GABA_A$ receptor (personal communication Prof. A.Draguhn, Heidelberg), but the responsible domain of APLP2 could not yet be specified. $GABA_A$ receptors are pentamers, composed of different subunits (α-γ) that exist in different isoforms. The α-subunit determines the kinetics of activation and inactivation and therefore the identity and composition of subunit isoforms influence the decay time of the synaptic current (Okada et al., 2000; Fisher, 2004). α1 subunit knock-out mice have smaller and less frequent, but prolonged mIPSCs, the subunit however was found predominantly on CA1 interneurons and less on pyramidal neurons (Goldstein et al., 2002). The net effect of these alterations on LTP of pyramidal neurons is too complex to be predictable.

The very moderate LTP deficit makes it unlikely that an additional role of APLP2 in LTP relevant functions prevented LTP deficits in mice expressing truncated APP but intact APLP2. It seems to be predominantly the APPsα domain of APP which mediates the effects ultimately resulting in LTP and learning deficits. To determine whether APLP2sα has only a supportive role in combination with APPsα or separate functions, e.g. in neurite outgrowth, expression of APLP2sα on an APP$^{-/-}$ background would be required.

Subsequent crossing of APLP2$^{-/-}$ mice with APP$^{-/-}$, APPsα or APPΔCT15 expressing strains allowed to investigate whether minor or complementary functions of APLP2 had compensated for the loss of APP in young APP$^{-/-}$ mice and in aged mice of the two strains expressing APP truncations. APLP2$^{-/-}$/APP$^{-/-}$ mice die shortly after birth, not allowing examination of age-dependent alterations in synaptic plasticity. Young adult mice expressing either of the two truncated APP versions on an APLP2$^{-/-}$ background show no deficit in LTP. Aged mice expressing the APPsα ectodomain of APP without APLP2 have not been tested yet, APPΔCT15$^{-/-}$ mice crossed with APLP2$^{-/-}$, however, did not display age-related differences in LTP levels, even when using very weak LTP induction protocols. So, the APPsα domain or the APPsα domain plus the transmembrane domain of APP were sufficient to rescue LTP in aged animals also under simultaneous lack of APLP2. Lack of the AICD again did not impair the intactness of synaptic plasticity in old age.

However, the fact that mice lacking both APP and APLP2 holo-proteins die shortly after birth whereas mice lacking only either one survive at Mendelian frequencies, conveys an additional role of the ectodomain either of APLP2 or of both together. *In vivo*, effects on neural migration and neurite outgrowth occur only during simultaneous lack of APP, APLP2 and APLP1 (Heber et al., 2000; Herms et al., 2004). For combined lack of APLP2 and APP, few histopathological or functional abnormalities have been described. Wang and colleagues reported defects of the neuromuscular junction, comprising aberrant apposition of pre- and postsynaptic markers with acetylcholine-receptors as well as reduced vesicle numbers in presynaptic terminals and reduced numbers of miniature endplate potentials (Wang et al., 2005). In a recent paper, a deficit in glutamatergic signal transmission in could be specified to reduced expression levels of the glutamate transporter VGLUT2. This deficits was rescued re-introduction of the AICD and ALICD by transfection (Schrenk-Siemens et al., 2008). But as mice lacking both AICD and ALICD are viable, the reduced VGLUT2 expression cannot be causal for the death of the double KO mice. Whether the NMJ-specific defect is causal for the premature death of APLP2$^{-/-}$/APP$^{-/-}$ mice could only be determined, if it was known whether it is absent in APPΔCT15/APLP2$^{-/-}$.

So, whereas for maintenance of synaptic plasticity in age, APLP2 plays only a minor role, perinatal lethality of APLP2$^{-/-}$/APP$^{-/-}$ mice, but not of APPsα-KI/APLP2-KO mice conveys a supporting or complementary role of the APLP2sα ectodomain of APLP2. The nature of the overlap and a specific role have not been clarified.

4.1.5 The impact of age-dependent processes

The prevalence and individual risk of Alzheimer´s Disease increase with old age. Similarly, $APP^{-/-}$ mice only develop deficits in old age. This raises the question whether the two phenomena have something in common and whether the deficit in aged $APP^{-/-}$ mice constitutes an aggravated form of aging or something mechanistically different. What $APP^{-/-}$ mice, healthy aged mice and mice expressing AD mutations share, is some form of age-dependent LTP impairment. It is undisputed that aging profoundly alters the mechanisms of synaptic plasticity, for a review see Burke and Barnes (2006). Mice also have differently reduced levels of APPsα.

The deficit in activity-dependent plasticity in aged $APP^{-/-}$ mice involved altered GABAergic signaling. Aging in general is associated with alterations in GABAergic signaling and with altered intracellular Ca^{2+} levels, but the mechanisms are under controversy. Also, AD is often regarded as an aggravated form of aging and said to affect both mechanims. The mice used here differ significantly from AD mouse models as they express neither of the cleavage products of APP, namely no Aβ. It therefore has to be discerned between models of explanation arguing with increased Aβ levels as such (as for example in cases of early FAD, where more Aβ 1-42 is produced) and those arguing with a generally increasing imbalance of cleavage products to the disadvantage of APPsα.
Whether the observed alterations in GABAergic signaling involve age-dependent alteration of GABA-receptor density or changed prevalence and/or activity of GABAergic neurons, my experiments could not specify. But they showed that not the existence of Aβ, but the lack of APPsα is the key regulator. During healthy aging, going along with lower levels of APPsα than young animals, reduced numbers of hippocampal interneurons were found to be compensated by up-regulation of the expression of the α1 $GABA_A$ receptor subunit (Vela et al., 2003). This constitutes the situation of wildtype mice in these experiments. To determine whether this phenotype was aggravated in $APP^{-/-}$ mice, levels of this subunit would have to be determined.

The mechanism of LTP induction may also be impaired independently from GABAergic signaling. Whereas most of the biophysical properties of neurons stay unaltered, aging goes in hand with increased Ca^{2+} conductance in neurons (Burke and Barnes, 2006). There is increasing evidence that Ca^{2+} dysregulation is also causally involved in the pathogenesis of AD: not only do all genes that increase the susceptibility to AD also modulate Ca^{2+} signaling, but Ca^{2+} dyshomeostasis is an early manifestation of the disease, as alterations in Ca^{2+} signaling occur already in the initial phase of the disease and even before development of overt symptoms and especially detectable Aβ pathology. This led to the formulation of the "Ca^{2+}-hypothesis", stating that sustained disturbances in Ca^{2+} homeostasis lead to accumulation of intracellular damages constituting the proximal cause of neurodegeneration in AD (Khachaturian, 1989). Such disturbances occur, in minor form, also during normal aging.

Apart from such damages, there is also a direct effect of alterations in Ca^{2+} buffering on LTP. In pyramidal cells of area CA1 and CA3, the expression of voltage-dependent L-type Ca^{2+} channels is upregulated, leading to higher levels of cytosolic Ca^{2+} by differential activation of release from intracellular stores. Higher $[Ca^{2+}]_i$ levels increase activation of outward K^+ currents after depolarizing stimuli, which enlarges afterhyperpolarisation (AHP) and results in decreased opening probability of the NMDA receptor and thereby the LTP threshold (Landfield and Pitler, 1984; Moyer, Jr. et al., 1992; Disterhoft et al., 1996), schematically depicted in figure 4.1, as adapted from Foster (2007). Elevated cytosolic Ca^{2+} levels also facilitate LTD (Norris et al., 1996); simultaneously, LTP by strong stimuli at the same time is not altered (Foster, 2007). This corresponds to a right shift of the stimulation-potentiation function in the model of Bienenstock, Cooper and Munro on plastic alterations of synaptic strength.

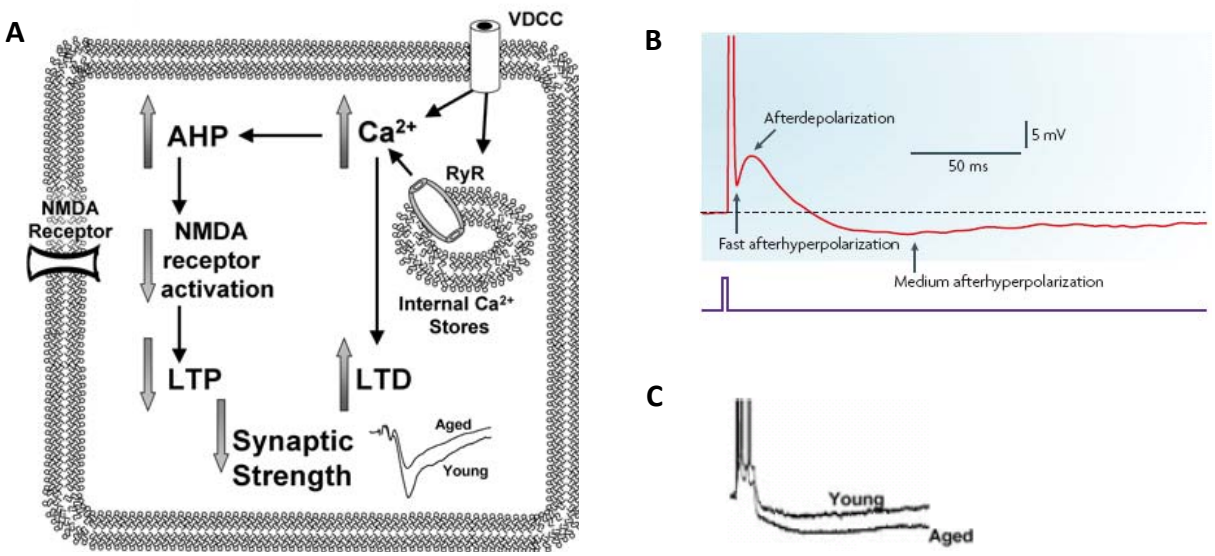


Fig. 4.1 (A) Modest tonic increase of cytosolic Ca^{2+} via voltage-dependent Ca^{2+} channels and from intracellular stores on the one hand increases after-hyperpolarisation (AHP, **B**) which raises the threshold for LTP by inhibiting activation of NMDA receptors and on the other hand promotes LTD. **(C)** In aged animals, the slow components of afterhyperpolarization are strongly increased. (A) and (C) adapted from Foster (2007), (B) from Bean (2007).

This mechanism is also regarded as contributing to the symptoms of AD. Altered processing of APP has been regarded as disrupting the ability of neuromodulators to regulate the AHP amplitude (Disterhoft et al., 2004). On the other hand, continuously elevated membrane potentials lead to slight tonic activation of NMDA receptors. This reduces the amplitude of rapid Ca^{2+} influx during LTP or learning stimuli and is regarded as one of the reasons why the NMDA receptor antagonist Memantine can ameliorate AD symptomatic (Frankiewicz and Parsons, 1999; Danysz et al., 2000).

I could not specify whether altered Ca^{2+} levels were also responsible for reduced LTP in aged $APP^{-/-}$ mice. Mostly, alterations of intracellular Ca^{2+} homeostasis have been ascribed to the APP intracellular domain or to Aβ, see review in Mattson and Chan (2003), but an older study reported the ability of APPsα to reduce cytosolic Ca^{2+} by activation of K^{+}-channels (Furukawa et al., 1996). This fits well with notions of increased excitotoxicity during aging and also with the model of shifted LTP and LTD thresholds. From my data no assertion on the causal relationship between GABAergic inhibition and Ca^{2+} levels can be inferred. An effect of dysregulated Ca^{2+} buffering is likely to equally affect GABAergic neurons. Having higher levels of tonic activity (Hammond, 2003), they are more vulnerable to excitotoxicity. Also, reduced tonic inhibition leads to higher resting membrane potentials. So, there are strong indications that both systems are affected by aging processes as well as by lack of APPsα.

Taken together, I found an age-dependent LTP deficit in mice lacking APP that is due to APPsα and involves dysfunction of GABAergic signaling. This is consistent with studies showing memory-enhancing effects of APPsα and with studies finding altered GABAergic signaling in aging as well as in AD. They extend those studies insofar as they provide a link between the two alterations and act in an in vivo model avoiding unphysiological concentrations as well as pathological conditions and distortion by other APP cleavage products. It had been proposed that compensatory mechanisms that may mask the APPsα deficit in young mice are lost during the aging process (Ring et al., 2007). A key candidate for a compensatory actor had been APLP2sα. I could now show that it is far less involved in the same processes. What was beyond the scope of my study was the causal link between APPsα and GABAergic dysfunction and the mechanism by which APPsα mediates this function. A plausible candidate is an effect either by or on Ca^{2+} homeostasis.
Two further conclusions can be drawn. A), also the lack of APPsα, typically experienced in AD has consequences for memory function, without ruling out the significance of deleterious effect of Aβ in the brain and b), alterations in a still non-pathological system can result in AD-like alterations without degenerative processes. This shows that neurodegeneration in AD is likely to be only a secondary effect concerning memory function.

4.2 The role of the APP and APLP2 intracellular domains in synaptic function

I also investigated the function of the intracellular domains of APP (AICD) and APLP2 (ALICD) in synaptic plasticity and synaptic function. Both have identical sequences and are set free by γ-secretase.

Examination of aged gene-targeted mice lacking a functional AICD revealed no alterations in either short- or long-term plasticity or in behavior (Fig. 3.2 and 3.3). To determine potential masking of effects by the ALICD, I extended my examination to strains, cross-bred from lines of mice expressing no functional AICD, with homozygous APLP2$^{-/-}$ mice. So, in both lines, internal littermate controls were APLP2$^{-/-}$/APP$^{+/+}$ animals.

In both of these strains I found several identical age-independent alterations. Short-term plasticity, measured as paired-pulse facilitation, was significantly enhanced in mice with truncated APP constructs, but partly normalized after LTP induction. After application of theta burst stimulation protocols for LTP induction, fEPSP slopes in mice expressing truncated versions of APP transiently decreased on average to half of the potentiation levels of wildtype animals for several minutes before recovering. Long-term plasticity, examined as LTP induced with different protocols did not reveal alterations. Mice of both strains also were hyperactive and showed circling behavior.

4.2.1 Long-term plasticity

I induced LTP in young-adult animals of both strains by a regular protocol and with a "priming" protocol, consisting of a two-step stimulation. Here, a weak LTP-inducing stimulus (one 100Hz theta burst stimulus train instead of three and using an fEPSP size of 30 % instead of 40% as baseline) was followed 30 minutes later by a regular one (3 stimulus trains). The weaker an induction protocol is, the higher is its sensitivity for detection of subtle deficits. The first, priming stimulation can detect more complex changes in the dynamic processes, alterations in the range of synaptic plasticity or possibly involving potentiation of inhibitory contacts. Recent prior activation has been reported to alter excitability and reactivity to further stimuli (Christie et al., 1995; Christie and Cameron, 2006; Mellentin et al., 2007). In APP overexpressing animals crossed with mice lacking β-secretase, selectively only the second potentiation was higher than in mice expressing β-secretase (Ma et al., 2007). However, neither after weak stimulation nor under conditions involving dynamic processes triggered by the first stimulation, a significant difference, as determined by student's t-test, was observed in either strain (Fig. 3.7). Long-term LTP requiring gene transcription was intact, too.

In these experiments, mice with neither AICD nor APLP2 were compared to APLP2$^{-/-}$ mice, which in old age have a moderate LTP deficit. So, there may be a mild additional deficit caused by the combined deletion. A tendency in this respect is provided by the significances yielded by ANOVA. However, a clear masking effect by the ALICD could be excluded.

This was unexpected, as several functions with potential relevance for LTP have been proposed for the APP and APLP2 intracellular domains AICD and ALICD, either for their roles as intracellular interaction domain of the holo-protein or for separate signaling properties of the cleaved fragment. It revealed that a signaling function, either for intracellular processes or as transcriptional regulator, as suggested by the proven adaptor protein interactions of the YENPTY motif, was not relevant for LTP.
Likewise, the lack of APP internalisation via the AICD as anchor did not result in improved LTP. Adaptor proteins link APP (and putatively APLP2) via the YENPTY motif to the low density lipoprotein receptor related protein LRP (Pietrzik et al., 2004). Genetic deficiency of LRP in LRP$^{-/-}$ cells increased APPsα production by reduction of endocytosis (Pietrzik et al., 2002). The YENPTY deletion was functional as mouse embryonic fibroblasts from mice carrying the APPΔCT15 deletion mutant of APP had slightly higher amounts of plasma-membrane bound APP, reduced Aβ production and increased rate of APPsα secretion (Ring et al., 2007). A reason for the unimproved synaptic plasticity may be that the amount of APPsα was still high enough in wildtype animals to fulfill all functions. But more importantly, the expression levels of APP in both knock-in strains were slightly lower than in WT animals (Ring et al., 2007), possibly compensating for the altered cleavage balance.

4.2.2 Short-term plasticity

Transient alterations of network responses to repetitive stimuli critically influence the integration and processing of information and therefore short-term modification of neuronal signaling is tightly regulated in the nervous system. During and after repetitive stimulation, neuronal activity in a population of synapses is transiently modulated in a predictable fashion (Stevens and Wang, 1995; Hjelmstad et al., 1997; Volianskis and Jensen, 2003). In both strains such short-term plasticity was affected in several aspects.

Paired-pulse facilitation (PPF) is a common form of short-term plasticity at the CA3-CA1 synapse and characterizes the gain in postsynaptic response to the second of two successive pulses within the range of tens of milliseconds. Examination of young adult animals of the APLP2$^{-/-}$/APPsα- and the APLP2$^{-/-}$/APPΔCT15 strain showed up to 30% higher

facilitation ratios than littermates with wildtype APP (Fig. 3.9). After LTP induction the effect became less pronounced.
Additionally, during the first minutes after LTP induction fEPSPs transiently decreased in mice expressing truncated APP constructs. They were minimal after 2.5 minutes, but reached potentiation levels comparable to wildtype levels after 10 minutes. The effect was present after 100Hz theta burst stimulation, but not after 100Hz stimulation with tetanus protocol and was more pronounced after 50 Hz theta burst stimulation (Fig. 3.10), falling even under baseline. As 50 Hz stimulation generally results in lower LTP levels, this gives the impression of two processes being overlayed in the fEPSP: a potentiation of excitatory synapses and another effect independent from LTP, either on the pre- or on the postsynaptic side. To my knowledge, such an effect has not been described yet.

These phenotypical characteristics strongly point to alterations in presynaptic functionality related to transmitter release, depending on the average release probability or the levels of cytosolic Ca^{2+} at the presynaptic terminal triggering exocytosis. But diverse contributions of inhibitory neurons modulating information transfer at excitatory-excitatory synapses and within neurons shape short-term plasticity. In the following, these components and their applicability to both phenomena will be discussed.

4.2.2.1 Presynaptic Calcium dynamics

At the inter-stimulus intervals investigated here, paired pulse facilitation of transmitter release is determined by the average vesicle release probability, the stimulus-dependent accumulation of Ca^{2+} in the presynaptic terminal and the speed at which Ca^{2+} is cleared from it (Zucker, 1999; Emptage et al., 2001). The vesicle release probability depends on the size and refilling speed of the readily releasable vesicle pool and the cytosolic Ca^{2+} concentration in response to an action potential (Cabezas and Buno, 2006). If vesicle availability is not the limiting factor, the response to the second stimulus is higher, because residual Ca^{2+} from the first depolarisation increases the amount of transmitter release.
The unaltered basal synaptic transmission in both mouse strains shows that general release probability, resting Ca^{2+} levels and the amount of Ca^{2+} influx from extracellular space in response to an incoming axonal signal were unaffected at the CA3-CA1 presynapse and only alterations of dynamic processes can account for the phenotype.

The dynamics of the presynaptic $[Ca^{2+}]_i$ levels critically determine the transmitter release. At rest, they are significantly lower than in the extracellular medium. The net increase depends on the amount and time scale of Ca^{2+} influx through voltage dependent Ca^{2+}-channels and the re-establishment of resting levels by clearance of Ca^{2+} from the cytosol (Fig. 4.2).

Ca^{2+} influx from the extracellular space into the cytosol of the axon terminal in response to an action potential follows activation of voltage-gated Ca^{2+} channels in the vicinity of docked vesicles in the active zone. The level of presynaptic Ca^{2+} is further increased by Ca^{2+} induced Ca^{2+} release from the endoplasmic reticulum (ER). Blocking of this mechanism reduces PPF (Emptage et al., 2001).

To ensure accurate signal transmission, the concentration of free cytosolic Ca^{2+} needs to be rapidly restored. This involves extrusion from the terminal, uptake into the ER or temporary clearance via buffering by proteins or mitochondria (Levy et al., 2003; Scott, 2007). Extrusion from of the cell is achieved by ATP-dependent Ca^{2+} pumps and Na^{+}-Ca^{2+} exchangers using the Na^{+} gradient. Transport into the ER and in second line also into mitochondria is achieved by the sarco-/endoplasmic reticulum Ca^{2+} ATPase (SERCA) pump, for a review see Blaustein and Golovina (2001). The mechanism by which Ca^{2+} release activates Ca^{2+} currents into the ER, is referred to as capacitative Ca^{2+} entry (CCE), and has been implicated in the pathogenesis of Alzheimers Disease (Smith et al., 2002; Giacomello et al., 2005).

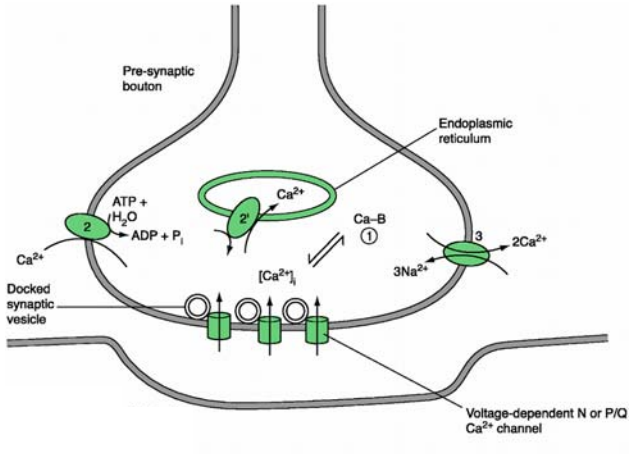


Fig. 4.2 Mechanisms of re-establishment of cytosolic Ca^{2+} levels after influx through voltage-dependent Ca^{2+} channels comprise inactivation by buffering (1), pumping into the ER or over the plasma membrane by Ca^{2+} ATPases (2) and transport out of the cell by Na^{+}-Ca^{2+}-antiports (3).
Adapted from Hammond (2003).

In both mouse strains, Ca^{2+} influx is not affected as shown by similar response amplitudes of the first response. Higher transmitter release in response to the second pulse, however, argues for decelerated Ca^{2+} clearance.

A multitude of studies support an effect on Ca^{2+} homeostasis. The majority, however, make use of mutations in PS1, the catalytic domain of the γ-secretase-complex. PS1 mutations can disturb Ca^{2+} homeostasis in a way that more Ca^{2+} is released from the ER upon stimulation (Mattson and Chan, 2003). Also, mutation of PS1 has been reported to alter capacitative Ca^{2+} entry into the ER, resulting in overfilled Ca^{2+} stores (Schneider et al., 2001; Herms et al., 2003). Leissring and co-workers observed that in fibroblast cultures from mutant PS1 transgenic mice stimulation of the IP3/Ca^{2+}-signaling cascade via a cell surface receptor resulted in higher quick increases, but lower sustained levels of cytosolic Ca^{2+}. Ablation of PS1, on the other hand, led to attenuated Ca^{2+} signals (Leissring et al., 2000; Leissring et al., 2002). Whereas the authors

assumed that alterations in mechanisms of Ca^{2+} release are mediated by the PI3/Ca^{2+}-signaling cascade and regulation of genes that are involved in Ca^{2+} homeostasis, Hamid and colleagues proposed an effect via mitochondrial dysfunction, hypothesizing that ultimately there is less ATP at hand for the SERCA pump (Hamid et al., 2007).
There is increasing evidence that the observed effects are at least partly due to the AICD and ALICD themselves, not to other substrates of γ–secretase. The same effects as caused by γ–secretase inhibition were also present in $APP^{-/-}$ cells and could be rescued by transfection with the AICD (Leissring et al., 2002; Herms et al., 2003; Hamid et al., 2007). Hamid and colleagues had found that the ER of $APP^{-/-}$ cells contained less Ca^{2+} whereas the cytosol contained more, which could be rescued by transfection with the AICD.

So, although some of the mentioned studies only refer to static alterations of Ca^{2+} levels, the AICD and ALICD seem to enhance release of Ca^{2+} from the ER upon stimulation, but also reestablishment of cytosolic resting Ca^{2+} levels, which is line with increased transmitter release during repetitive stimulation in my experiments. Concerning the mechanism of the altered dynamics, there is no consensus.
Unfortunately, all studies specifically on AICD function have been performed in cell culture of nonneural cells. The enhanced paired pulse facilitation now shows that the alterations extend to neurons and have functional consequences. Direct evidence, however, that altered Ca^{2+} homeostasis is involved, yet has to be provided by monitoring the Ca^{2+} elevation in response to pulse pairs.
To strengthen the hypothesis I provided here, it may also be promising to investigate hair cells from the inner ear. Here, a very steep Ca^{2+} gradient exists over the plasma membrane. The striking circling behavior of mutant mice in both strains hints to a defect of the vestibular system, part of the inner ear, which can possibly be attributed to dysregulated Ca^{2+} levels in hair cells.

4.2.2.2 Neurotransmitter vesicle pools

Mechanisms of vesicle “logistics” may account for the transient decrease of fEPSP size after induction of LTP. Neurotransmitters are stored in and released from presynaptic vesicles which are organized in sequentially arranged pools. The release is effected from the smallest, the readily releasable pool (RRP), consisting of vesicles that are docked at the active zone in close vicinity to Ca^{2+} channels. In hippocampal boutons this pool is not depleted by pairs of stimuli, but during the course of tetanic or theta burst stimulation (Schikorski and Stevens, 2001). It is refilled mostly from the so-called recycling pool, providing vesicles during moderate physiological stimulation and being loaded by continuous recycling of vesicles (Harata et al., 2001), for a review see Rizzoli and Betz (2005). Transmitter release from docked vesicles is

effected by Ca^{2+} sensitive membrane fusion proteins transiently fusing plasma and vesicle membrane, reviewed in Brose (2008).

An explanation based on depletion of vesicle pools, has to explain several aspects of the transient decrease of fEPSPs. Firstly, it has to account for the specificity of the phenomenon for theta burst stimulation. Although the total increase of presynaptic Ca^{2+} might be similar, the kinetics of Ca^{2+} influx are different for theta burst stimulation (TBS) and tetanus, because of the intermittent pauses of stimulation in the TBS protocol and the relocation of vesicle between pools may also be Ca^{2+} dependent.
Secondly, it has to explain how fEPSPs during the first seconds after TBS could be still potentiated. The phenomenon of post-tetanic potentiation, mediating the steep increase during the first seconds after LTP-induction is also presynaptically determined, as it arises from a transient presynaptic cytosolic Ca^{2+} concentration due to slow efflux of accumulated Ca^{2+} from buffer systems (Volianskis and Jensen, 2003). A possible explanation is that this increased Ca^{2+} mobilized the "last" vesicles, before regular levels were established again. This would also fit with the observation that such an elevation was not present immediately after a 50 Hz theta burst stimulation, as here less Ca^{2+} accumulates.
Furthermore, the phenotype was not reproducible in aged APPΔCT15 expressing animals (Fig. 3.10). After induction of a 50 Hz TBS there was a lack of post-tetanic potentiation in both genotypes, but in none of the single experiments on KI animals there was an additional collapse of the EPSP as observed in young animals. The reasons can only be speculated on. Generally, the amounts of transmitter release are lower in aged animals, so vesicle pools might not be depleted completely. Also, altered Ca^{2+} levels in aged animals can influence the mentioned factors in complex ways, e.g. preventing run-down of the RRP due to reduced transmitter release.

A recent paper reported altered loading of vesicles with neurotransmitter molecules in $APLP2^{-/-}/APP^{-/-}$ mice (Schrenk-Siemens et al., 2008). In pyramidal neurons generated from embryonic stem cells, the authors found down-regulated expression of the vesicular glutamate transporter VGLUT2 and reduced uptake and/or release of glutamate. They could create the same phenotype in cells from wildtype mice by blocking γ-secretase activity and rescue it in cells from $APLP2^{-/-}/APP^{-/-}$ mice by re-introduction of a construct of the transmembrane domain and AICD of human APP. This indicates a role of the AICD and ALICD in controlling VGLUT2 expression. VGLUT2 is expressed during development and later replaced by VGLUT1 (Wojcik et al., 2004), which may explain why only 20% of KI animals survive through weaning. Interestingly, they also observed smaller fEPSPs in organotypic cultures of $APLP2^{-/-}/APP^{-/-}$ mice, whereas I found identical levels of fEPSP sizes in knock-in and wildtype littermates. The effect might not have been visible in the mice I examined, because I only experimented on the 20% of APPΔCT15 expressing animals that survived through weaning. But the smaller fEPSP

sizes may also have been an effect of the simultaneous lack of APPsα and APLP2sα, resulting in reduced axonal outgrowth and thus not linked to the AICD and ALICD.
Two studies by Ho and co-workers investigated the AICD adaptor proteins Mint1 and 2 (X11α and β), which act on synaptic vesicle release via the membrane fusion protein Munc18A (Hata et al., 1993). Knock-out mice showed presynaptic functional deficits, namely reduced vesicle release resulting enhanced PPF and decreased PPD, depending on whether the isoform predominant on excitatory neurons or inhibitory neurons was knocked out (Ho et al., 2003; Ho et al., 2006). But here, the identical sizes of postsynaptic responses to single pulses and even elevated responses to paired pulses indicate that the transmitter amount in vesicles and the immediate availability of vesicles in the RRP was not altered. However, altered kinetics for filling of vesicles and vesicle pools may have become relevant after tetanic stimulation and might account for the transiently decreased fEPSPs after TBS.

So, from earlier studies on APP, APLP2 and related proteins no hypothesis about alterations of vesicle and vesicle pool filling can be inferred. To determine whether such mechanisms contributed to this phenotype, changes in stimulated vesicle release and refilling of vesicle pools would have to be evaluated by whole cell measurements of use-dependent depression following high-frequency stimulation. To determine alterations in vesicle filling amplitudes of miniEPSCs would have to be determined.

4.2.2.3 GABAergic inhibitory synaptic transmission

Schaffer Collateral axons from CA3 pyramidal neurons innervate several types of GABAergic interneurons in distinct regions of the CA1 *stratum radiatum* (Altman et al., 1973). The transmitter GABA acts via ionotropic $GABA_A$ receptors, associated with Cl^--channels that mediate quick point-to-point inhibition ($IPSP_A$) and via G-protein coupled metabotropic $GABA_B$ receptors mediating hyperpolarisation by inactivation of voltage-dependent Ca^{2+}-channels and K^+-channels ($IPSP_B$) (Hammond, 2003).

Some studies reported alterations of GABAergic signaling in connection with the AICD and ALICD. Knock-out mice for Mint1, an adaptor protein of the AICD and ALICD that binds to the vesicle function protein Munc18A have altered GABAergic and glutamatergic short-term potentiation due to reduced transmitter release (Ho et al., 2003; Ho et al., 2006). In mutant PS1 mice producing larger amounts of AICD and Aβ, alterations in the temporal composition of cortical EEG oscillations were found (Wang et al., 2002). As this rhythmic activity is mediated by phase-locked activity of inhibitory neurons, this indicates altered GABAergic activity. In the same mouse mutant, also age-independent auditory gating was observed (Wang et al., 2003). Sensory gating modulates the processing of the second in a succession of two stimuli, involving

GABAergic feed-back and feed-forward inhibition and is regarded as being hippocampus-based (Miller et al., 1995). Here, though, alterations of GABAergic contributions cannot primarily account for the effects.

Paired-pulse facilitation at all tested frequencies is shaped by different components of GABAergic synaptic transmission. GABAergic IPSPs are biphasic, comprising a quick $GABA_A$-mediated hyperpolarisation that peaks after 5-15 ms and is decayed after 10-50 ms (Maccaferri et al., 2000) and a $GABA_B$-mediated current peak after 130 – 300 ms decaying over the following 400 – 1300 ms (Hammond, 2003), for an example see figure 4.3.

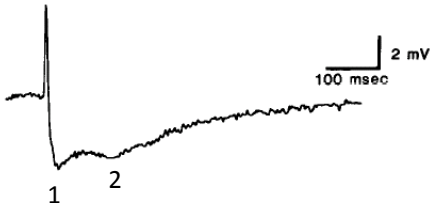


Fig. 4.3 GABAergic IPSPs on pyramidal neurons are biphasic, comprising a quick $GABA_A$ mediated (1) and a slower $GABA_B$-mediated (2) component. Adapted from Kloosterman et al. (2001).

GABAergic neurons excited by Schaffer collateral axons together with CA1 pyramidal neurons form feed-forward inhibitory synapses onto somata and dendrites of CA1 neurons truncating the EPSP. Reduction of GABAergic synaptic activity results in prolonged depolarization of the pyramidal neuron and the amplitude of a second EPSP in this time window will add up with the existing depolarization. Its slope will also steepen, because the somatodendritic information transmission by passively travelling depolarization is shunted to a lesser extent by nearby active GABAergic synapses. $GABA_B$ receptors additionally facilitate the second EPSP at longer inter-stimulus intervals by autocrine feedback inhibition of GABA release (Davies and Collingridge, 1996). This results in smaller $IPSP_A$s in response to the second pulse (so-called paired-pulse depression), additionally widening the second EPSP.
Therefore, in sum, several components contribute to paired pulse facilitation at different frequencies. At 50 - 100 Hz, presynaptic facilitation of transmitter release is maximal (Clark, 1994). At very high frequencies (10 ms ISI, equaling 100Hz), the EPSP facilitation nevertheless is slightly lower, because it is shunted by the monosynaptic $GABA_A$-mediated IPSP (Nathan and Lambert, 1991). At frequencies of 50 Hz (equaling a 20 ms ISI), presynaptic facilitation of transmitter release makes the largest contribution and at lower frequencies, mainly $GABA_B$-mediated heterosynaptic depression depression of $GABA_A$ IPSPs triggered by the first pulse enhances the second EPSP (Nathan et al., 1990; Davies and Collingridge, 1996).

In experiments on both mouse strains expressing APP truncations, the differences in facilitation ratios caused by the genotype difference were largest at 100 and 50 Hz. This argues for predominant alterations in presynaptic, glutamate-release related mechanisms at these

frequencies. Indirect testing revealed that GABAergic contributions at lower frequencies also were not significantly changed. The alteration of GABAergic activity is often tested by examining paired-pulse depression (PPD) of IPSPs. Here, this was done indirectly by measuring the influence of PPD on fEPSP widths at the inter-stimulus interval of 160 ms. The width of the second EPSP was not altered in either of the tested strains (Fig. 9 C, F), indicating that feedback suppression of GABA release and also indirectly GABA release itself were not affected, however the examination of field potentials here demands robust alterations. The relative increase in facilitation between the genotypes was largest at 100 and 50 Hz (table 3.1), indicating a predominant influence of presynaptic accumulation of $[Ca^{2+}]_i$.

The transient decrease of fEPSPs suggests involvement of GABAergic signaling, because it occurs selectively after theta burst stimulation (Fig. 3.11). IPSPs of $GABA_A$ and $GABA_B$ receptors can also be potentiated in an NMDA receptor dependent way, however only by theta burst, not by tetanic stimulation, due to different kinetics of $[Ca^{2+}]_i$ elevation at the pyramidal neuron (Perez et al., 1999). However, there is no consistent explanation why such a potentiation should only last only for several minutes. If re-establishment of $[Ca^{2+}]_i$ was prolonged in presynaptic terminals, this could also lead to transient increases of GABAergic activity. Yet this hypothesis has to remain speculative, because no assertion can be made as to why the effect should last for minutes instead of seconds. Also, in aged APPΔCT15 expressing animals, the phenotype was not reproducible (Fig. 3.10). After induction of a 50 Hz TBS there was a lack of post-tetanic potentiation in both genotypes, but in none of the single experiments on KI animals there was an additional collapse of the EPSP as observed in young animals. There are no indications for a specific change of GABAergic signaling in aged transgenic animals.

So, although GABAergic contributions to short-term plasticity are crucial and generally altered mechanisms of transmitter release will affect inhibitory neurons as well as excitatory neurons, they can be excluded as the only or primary cause of the phenotype, as alterations of short-term plasticity were present also under circumstances that involve GABAergic signaling to a lesser extent. Gross alterations in inhibitory synaptic activity should also have resulted in altered LTP.

4.2.3 Long-term alteration of short-term plasticity

After LTP induction, the difference in paired pulse facilitation between mice expressing APP constructs and WT APP decreased. Facilitation rates were significantly reduced at an

inter-stimulus interval of 20 ms, albeit still larger than in animals with WT APP, which constitutes a long term effect on short-term plasticity 60 minutes after theta burst stimulation.
Also here, most likely presynaptic mechanisms were affected, because alterations occurred at a stimulus interval at which presynaptic contributions by altered glutamate release contribute to most of the effect.
LTP induction at the CA3-CA1 synapse is postsynaptic, but expression mechanisms include presynaptic aspects mediated by retrograde messengers. Such an alteration after LTP induction would represent a form of metaplasticity, if in an autoregulatory process synapses compensated for the malfunctioning. Differential potentiation of interneuron activity could also result from potentiation of excitatory synapses on interneurons or from potentiation of inhibitory synapses on CA1 pyramidal neurons. However, both phenomena would require differential alteration of postsynaptic $[Ca^{2+}]_i$, in tested and control animals and the identical rates of fEPSP potentiation count against such an alteration.

Summing up this chapter, the intracellular domains of APP and APLP2 are important for survival and critical for synapse function independently from aging processes. They have at least partially redundant functions, as only simultaneous lack of both proteins causes phenotypical alterations. The majority of alterations are of short-term nature, indicating that postsynaptic components of LTP in excitatory pyramidal neurons are not impaired. The present data indicate primary involvement of affected Ca^{2+} homeostasis. Both altered $[Ca^{2+}]_i$ levels and changes in vesicle release affect inhibitory neurons as well. However, alterations of inhibitory contributions seem to be of lesser relevance. The least explicable phenotype is the transient collapse of EPSPs after LTP induction. Here, all indications point to changes in transmitter release, especially as it was largest at the stimulation frequency affected most by altered transmitter release.
To specify the respective contributions and possible systems of interaction leading to the phenotype in mice lacking the AICD and ALICD, further experiments are necessary. To exactly determine the amount of involvement of GABAergic synaptic transmission, recording of PPF in the presence of $GABA_A$ and $GABA_B$ receptor antagonists or intracellular recording of PPD is necessary. To specify whether the effect of “post-tetanic depression” is an after-effect of alterations in presynaptic $[Ca^{2+}]_i$, theta burst stimulation will have to be presented in the presence of the NMDAR antagonist APV, blocking all postsynaptic effects of potentiation and enabling to measure purely presynaptic and Ca^{2+}-dependent post-tetanic potentiation. Separation of the contributions of altered Ca^{2+} homeostasis and transmitter release as such can only be accomplished by Ca^{2+} imaging and vesicle pool depletion experiments.

4.3 Involvement of APP in negative synaptic plasticity

In my investigation of the roles of APP in activity-dependent positive synaptic plasticity, i.e. enhancement of synaptic responses, I had found involvement of APPsα, the product of the α-secretase pathway, to be indispensable. If APP is subjected to the amyloidogenic cleavage pathway instead, Aβ and the AICD are released. The two cleavages are mutually exclusive and the equilibrium between them is affected by neuronal activity (Nitsch et al., 1993; Buxbaum et al., 1993; Lammich et al., 1999; Kamenetz et al., 2003). This suggests the question whether Aβ, as it is the only fragment produced exclusively in the amyloidogenic pathway, also serves a function in synaptic plasticity.

My experiments on LTP had excluded a role of Aβ on LTP under physiological conditions. But this did not preclude a specific effect on negative plasticity. Negative plasticity here is conceived as weakening of synaptic transmission, measurable as decrease of fEPSP size. There exist several molecules that are involved in negative, but not in positive synaptic plasticity. One of them is the pan neurotrophin receptor p75 ($p75^{NTR}$), also known to bind Aβ (Yaar et al., 1997; Kuner et al., 1998; Yaar et al., 2002). A role of Aβ exclusively in negative synaptic plasticity would argue for a specific physiological action of Aβ instead of an unspecific toxic one. Therefore, I examined long-term depression (LTD) of fEPSPs in its function for targeted loosening of synaptic contacts in the context of learning, memory performance and fine-tuning of neural networks. Apart from the neurotoxic properties of aggregated Aβ, also certain forms of soluble Aβ oligomers have been shown to affect synaptic transmission. But although a physiological role of Aβ under certain circumstances had been hypothesized, no experimental evidence under physiological conditions had been provided. My experimental model mimicked the physiological situation as close as possible, comparing wildtype animals to animals lacking Aβ and inducing LTD by synaptic, not by chemical stimulation.

I first examined two forms of LTD in juvenile $APP^{-/-}$ mice. One depends on activation of NMDA receptors and like LTP is expressed by changes in AMPA receptor properties, the other is induced via pre- and postsynaptic metabotropic glutamate receptors (mGluRs) and predominantly presynaptically expressed, therefore involving different mechanisms than LTP; for an extensive review see Kemp and Bashir (2001). In juvenile $APP^{-/-}$ mice, no alterations in LTD were found, independently from the protocol (Fig. 3.12). To exclude overlay of an adverse effect by the simultaneous lack of positive, i.e. putatively LTD inhibiting actions mediated by APPsα, I repeated my investigation of NMDA receptor dependent LTD in juvenile individuals of a mouse strain in which both wildtype and transgenic animals still express APPsα, using $APLP2^{-/-}$/APPsα knock-in mice. These also had unaltered LTD (Fig. 3.13 A, C).

These results seem to oppose the majority of studies on the role of Aβ in synaptic plasticity, reporting weaking of transmission and destabilisation of contacts. Addition of exogenous Aβ had been shown to promote NMDA receptor endocytosis (Snyder et al, 2005),

depress synaptic currents via action on AMPA and NMDA receptors (Kamenetz et al, 2003) and induce NMDA dependent degradation of PSD-95 (Roselli, 2007). Transfection with Aβ was reported to affect mGluR-dependent LTD and decrease the number of dendritic spines (Hsieh et al, 2006) and very recently Aβ oligomers from AD patients were reported to enhance mGluR-dependent LTD and promote spine loss via NMDA receptors (Shankar et al, 2008). However, these studies differed from my experimental approach in several respects.

Firstly, most of them selectively investigated the effect of the aggregation-prone $A\beta_{1-42}$ species, not $A\beta_{1-40}$, which is soluble under physiological conditions. Snyder and colleagues could show that $A\beta_{1-42}$ binds to postsynaptic α7-nicotinic acetylcholine (nACh) receptors and via activation of the protein phosphatase PP2B (calcineurin) and the tyrosine phosphatase STEP leads to endocytosis of the NR2B subunit of the NMDA receptor (Snyder et al., 2005) (Fig. 4.4). This neurotoxic action of the $A\beta_{1-42}$ species is now undisputed and signaling of these Aβ oligomers via calcineurin was recently confirmed (Reese et al., 2008).

In the intact organism, i.e. without genetic mutations leading to FAD and without aggravated forms of aging, $A\beta_{1-42}$ account for less than 10% of the Aβ (Selkoe and Podlisny, 2002) and still AD like pathology seems to be a common consequence of aging, which points a role of also of $A\beta_{1-40}$. Accordingly, Snyder et al. could not block NMDA receptor internalisation completely by blockade of the nACh receptor.

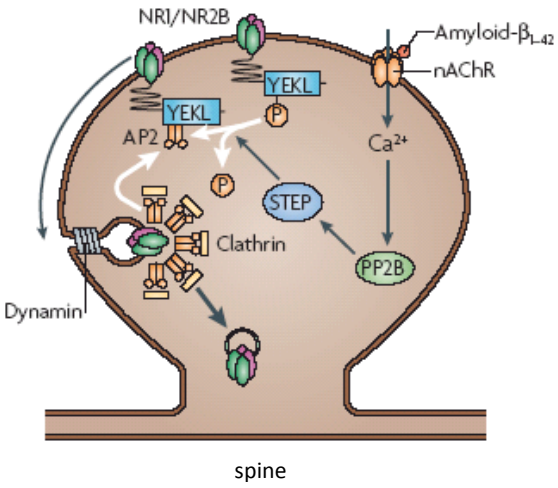


Fig. 4.4 $A\beta_{1-42}$ leads to internalisation of NMDA receptors. Binding of $A\beta_{1-42}$ to the nicotinic acetylcholine receptor (nAChR) activates the Ca^{2+} sensitive phosphatase PP2B which then activates the phosphatase STEP, dephosphorylating the NR2B subunit and promoting its internalisation. Adapted from Lau and Zukin (2007).

A second important difference of the studies mentioned above to my experimental paradigm is that whereas I compared lack of Aβ to physiological concentrations of endogenous Aβ, those examined conditions of surplus Aβ, either by investigating AD mutations or by exogenously adding or transfecting with Aβ. This increased the probability of unspecific, cytotoxic effects, because the higher the concentration of Aβ, the more likely aggregations are to occur. Aβ fibrils and other large aggregates are known to have toxic properties (Walsh et al., 2002b). This makes these experiments an adequate model for AD. They do not, however, allow

conclusions on putative functions of Aβ in the healthy organism. Therefore, from such studies it cannot be inferred whether soluble amyloid peptides have physiological relevance.
Thirdly, all studies, although working in the rodent system, applied human Aβ. Rodents naturally do not develop amyloid plaques, most likely, because human Aβ differs from the murine form in three amino acids which leads to two related consequences: murine Aβ might be less prone to form aggregates, however this is not well explored, and the altered amino acids might lead to different kinetics of Aβ degradation. The Aβ degrading enzyme, neprilysin (Iwata et al., 2001; Eckman et al., 2006) is downregulated with age (El-Amouri et al., 2008) and cleaves Aβ in the region that differs between humans and rodents. Both phenomena together decrease amounts of Aβ, thereby decelerating aggregate formation, and lead to the absence of plaques in rodents. Interestingly, in APP mutations leading to early-onset AD, the site of mutation lies either at the cleavage position of the γ-secretase or within the same three amino acids. Accordingly, one theory about the reason for late-onset AD is that this balance between anabole and catabole processes is disturbed (Yasojima et al., 2001; Iwata et al., 2002; Carpentier et al., 2002). This shows that the studies mentioned above are limited to the role of human Aβ in pathological situations. However, also murine Aβ can play a role, as demonstrated by the fact that aged neprilysin knock-out mice, experiencing a situation of overexpression of murine Aβ, have behavioral deficits and amyloid deposits (Madani et al., 2006). So, none of the studies examined the role of endogenous Aβ for physiological functions in the intact organism.

Simultaneously, my experimental setup contained two caveats that may have made the induction of an effect more difficult.
A consequence of a low basal concentration of Aβ may be that it only becomes effective after at least transient elevation of the levels of Aβ. A theory proposes that increased neuronal activity enhances Aβ production which then in a negative feedback loop depresses synaptic transmission during subsequent synaptic activity and NMDA receptor activation (Kamenetz et al., 2003). Later, activation of NMDA receptors was found to decrease α-secretase activity (Lesne et al., 2005). This could constitute the implementation of a homeostatic mechanism to control synaptic strength. An experiment to test that hypothesis would be to induce LTP by a strong protocol and shortly later begin NMDA receptor dependent LTD induction, a procedure called depotentiation.
The second difficulty might be constituted by the age of the tested animals. The effect of Aβ as such is not age-dependent, as for example acute application of Aβ can cause disruption of learned behavior without causing neurodegeneration (Cleary et al., 2005). But possibly, in juvenile animals the endogenous production is too low to yield effects in this experimental preparation. β-secretase activity is known to increase with age (Fukumoto et al., 2004), its prevalence during late development is not known. This could also be tested in a depotentiation experiment in aged animals.

A physiological role of Aβ may nevertheless be mediated via the $p75^{NTR}$. This receptor is a counterplayer of the growth and plasticity promoting neutrotrophin receptors TrkA, B and C, and mediates apoptotic signaling and, interestingly, also LTD (Rösch et al., 2005). It binds pro-neurotrophins, but also other molecules, among them Aβ (for reviews see Dechant and Barde (2002) and Lu et al. (2005). As Aβ is a ligand for $p75^{NTR}$, induction of LTD via $p75^{NTR}$ might constitute a physiological action of Aβ in the service of homeostatic maintenance of synaptic strength.

Intriguingly, aging not only activates the generation of Aβ by upregulation of β-secretase activity (Fukumoto et al., 2004), but also the expression of $p75^{NTR}$ (Costantini et al., 2006). It was shown that these two events are linked, as the aging-related switch from TrkA to $p75^{NTR}$ activates Aβ generation by molecular stabilization of the β-secretase (Puglielli et al., 2003; Costantini et al., 2005b), an effect that was abolished in $p75^{NTR}$ knock-out mice (Costantini et al., 2005b). At the same time $p75^{NTR}$ signaling also seems to be affected by Aβ. Whereas aggregated Aβ triggered cell death involving $p75^{NTR}$ activation, $p75^{NTR}$ was observed to have neuroprotective properties, saving from cell death induced by soluble Aβ (Costantini et al., 2005a). Importantly, direct interaction by binding of Aβ to $p75^{NTR}$ has been postulated, because the induction of apoptosis by Aβ was not observed in $p75^{NTR}$ knock-out mice (Yaar et al., 1997; Sotthibundhu et al., 2008) or after blocking of the $p75^{NTR}$ binding site by a protein mimicking Aβ (Yaar et al., 2007), but after application of $Aβ_{1-40}$ or $Aβ_{1-42}$ (Yaar et al., 1997; Sotthibundhu et al., 2008).

I performed experiments in a time frame when $p75^{NTR}$ expression is still elevated, before decreasing to levels found in the adult nervous system, possibly because during that time increased fine-tuning of the young neural net takes place. Aβ has not yet been reported to be higher during development. There seems to be a linear age-dose relationship in spite of upregulated $p75^{NTR}$. So, a putative signaling pathway of Aβ via $p75^{NTR}$ may mainly be active in aged animals, when $p75^{NTR}$ expression is upregulated again. Here, action of Aβ on $p75^{NTR}$ should be expected, but future experiments have to show whether such action would show in increased LTD.

In summary, the fact that LTD was completely unimpaired in juvenile mice lacking Aβ, asks for a redefinition of the role of Aβ. My experiments suggest the conclusion that in physiological concentrations in the healthy organism, it has no function in the induction and maintentance of LTD. This implicates that studies on pathophysiological processes using models of AD like symptoms do not allow inferences on the physiological situation.

4.4 Conclusion and outlook

This work has investigated the physiological role of APP, its active fragments and its homologues in synaptic function and plasticity. Its results emphasize that APP holds a much more prominent role than only giving rise to amyloid plaques, having profound effects on synaptic and network functions in the intact organism. By investigating different combinations of deletion mutants I was able to derive information about all major cleavage products of APP and about the interaction of APP and APLP2.

Concerning Alzheimer's Disease, my work supports the notion that imbalance of APP cleavages may be an early and critical cause and that its consequences might be more severe than elevated Aβ levels. APP and APLP2 have various effects on processes that are crucial for information processing and memory function, mediated separately by their cleavage products. So, the pathogenesis of AD has to be at least as complex as the functions of these fragments. All of them affect Ca^{2+} homeostasis and signaling in neurons. Exact maintenance of Ca^{2+} homeostasis is vital for intact function of neurons in a network, in terms of controlled spatio-temporal signaling. Ca^{2+} also mediates a multitude of signals for intracellular processes. Collectively, the available data show that perturbed cellular Ca^{2+} homeostasis plays a prominent role in the pathogenesis of AD, not only regarding neurodegenerative processes, but also altered signaling properties and activity-dependent synaptic plasticity. Given the strong effect of lack of the APP intracellular domain on short-term plasticity, such alteration of cleavage are prone to affect synaptic function.
This allows the conclusion that the necessary simultaneous dysregulation of all cleavage products leads to a combination and probably interaction of deleterious effects. With regard to the genesis of Alzheimer's Disease, this may, together with other aggravating factors, result in irreversible perturbation of crucial aspects of synaptic function in the hippocampus.

From the results of this study of synaptic function of APP and its homologues, two major topics emerged. One is the relevance of APP for neuroprotection in the context of aging mediated by the secretable APPsα domain. The other refers to alterations of Ca^{2+} buffering and network function, possibly as a consequence of altered Ca^{2+} homeostasis. Both open up avenues for further research.
A large step in understanding of the physiological function of APP will be gained by determination of the inter- and intracellular mechanisms of action of APPsα and its receptor. This will also elucidate the mechanisms by which APP preserves healthy aging. Also, the way by which the intracellular domains of APP and APLP2 mediate their intracellular effects on Ca^{2+} homeostasis is still open. The most promising follow-up experiment will be to investigate mechanisms of Ca^{2+} buffering in mice with combined deletions of APLP2 and APPΔCT15.

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Frequently used abbreviations

(f)EPSP	(field) excitatory postsynaptic potential
ACSF	artificial cerebrospinal fluid
AD	Alzheimer`s Disease
AICD	APP intracellular domain
ALICD	APLP intracellular domain
APLP	Amyloid Precursor Protein-like Protein
APP	Amyloid Precursor Protein
APV	2-amino-5-phosphopentanoic acid
CA	cornus ammoni
GABA	gamma-aminobutyric acid
KI	knock-in
KO	knock-out
LTD	long-term depression
LTP	long-term potentiation
PPF	paired-pulse facilitation
PS 1/2	Presenilin 1/2
PTX	Picrotoxine

Curriculum Vitae

25 April 1974	Born in Stuttgart, Germany
1993	Abitur (High School Exam), Königin-Charlotte-Gymnasium, Stuttgart
1993 - 2000	Studies of Law at the University of Tübingen
1996	Erasmus scholarship
	Studies of Criminology and Penal Law at the University of Sheffield, completed with thesis
	Title "Rising crime rates? Explanations of changes in Eastern and former Eastern European countries"
February 2000	Erstes Juristisches Staatsexamen (final academic law exam) of the state of Baden-Württemberg
April - Sept. 2000	Juristischer Vorbereitungsdienst (postgraduate judicial traineeship) at the district court of Heilbronn, Baden-Württemberg
2000 - 2005	Studies of Biology at the University of Tübingen
2002	Vordiplom (Bachelor`s Degree) in Biology
Oct. 2004 - Sept. 2005	Diploma Thesis at the Hearing Research Center Tübingen, supervisors Prof. Dr. M. Knipper and Prof. Dr. W. Schmidt
	Title “Alteration of behavior and responses of auditory neurons after application of salicylate in an animal model for Tinnitus”
September 2005	Diploma in Biology at the University of Tübingen (major subject: animal physiology, minor subjects: pharmacology, cellular biology)
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